From the Department of Molecular Medicine and Surgery

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VASCULAR REMODELING IN PULMONARY HYPERTENSION

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Cover image: Murine pulmonary arteriole (C57BL/6 mouse) examined by transmission electron microscopy. The right panel is an enlargement of the square in the left panel. M: mural cell; EC: endothelial cell. The images were kindly provided by Kjell Hultenby at Karolinska Institutet.

Vascular Remodeling in Pulmonary Hypertension

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VASCULAR REMODELING IN PULMONARY HYPERTENSION

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Stockholm 2015

To my wonderful family and friends

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

ABSTRACT

Pulmonary hypertension (PH) is a progressive obliterative vasculopathy with multifactorial etiology and high mortality rate. Endothelial dysfunction, excessive vascular remodeling, inflammation and *in situ* thrombosis contribute to increased vascular resistance and gradual occlusion of pulmonary vessels. Sustained high pressure in the pulmonary circulation increases right heart afterload and ultimately results in right heart failure. Current pharmacological treatments, like endothelin receptor antagonists, prostacyclin analogues, and phosphodiesterase-5 inhibitors, are primarily vasodilators and exert limited effects on vascular remodeling. Survival has improved, but far from enough, and the demand for lung transplantation has not been reduced.

Excessive growth of pulmonary artery smooth muscle cells (PASMCs) contributes to intimal hyperplasia, medial wall thickening, and plexiform lesions, the main features of vascular remodeling in PH. It is therefore important to explore factors which can control PASMC growth in order to identify potential targets for regression of PH.

Here, the roles of growth factors and proteoglycans in vascular remodeling were investigated in animal models of PH as well as in tissue specimens from patients.

In a rat model of PH associated with congenital diaphragmatic hernia, up-regulation of PDGF-B was found. Prenatal PDGF-B inhibition by imatinib, a tyrosine kinase inhibitor, was demonstrated to inhibit PASMC proliferation and to reduce pulmonary vascular remodeling in fetal rat lungs.

In a murine model of hypoxia-induced PH, increased perlecan deposition was observed. Genetically modified mice expressing heparan sulfate-deficient perlecan developed less PH, accompanied by decreased pulmonary vascular remodeling and reduced right ventricular hypertrophy. In addition, defective mural cell recruitment in pulmonary vessels was observed in heparan sulfate-deficient mice. A possible mechanism is impaired interaction between FGF-2 and its receptor and a pro-proliferative function for perlecan heparan sulfate in pulmonary vessels. The role of PDGF-B retention motifs, the C-terminal region of PDGF-B that interacts with heparan sulfate, was also explored. Hypoxia-induced PH was reduced in PDGF-B retention motif knockout, possibly due to defective retention of PDGF-B in the extracellular matrix and disturbed recruitment of mural cells.

Metabolic labeling experiments showed that a majority of the proteoglycans secreted by human PASMCs are decorated with chondroitin sulfate. Lung tissue sections from patients with PH revealed versican accumulation in vascular lesions. *In vitro*, mechanical strain and hypoxia increased versican production in PASMCs. The results suggested that versican is a pro-proliferative element in the vascular remodeling of PH.

In summary, this thesis work emphasizes the role of growth factors and proteoglycans, and interactions between the two, in the regulation of PASMC growth during PH development. Inhibition of growth factor activity or specific targeting of important functional domains of proteoglycans may be effective anti-remodeling strategies for future PH treatment.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Chang YT**, Ringman Uggla A, Österholm C, Tran PK, Eklöf AC, Lengquist M, Hedin U, Tran-Lundmark K, Frenckner B.
Antenatal imatinib treatment reduces pulmonary vascular remodeling in a rat model of congenital diaphragmatic hernia.
American Journal of Physiology. Lung Cellular and Molecular Physiology. 2012;302:L1159-1166

II. **Chang YT**, Tseng CN, Tannenberg P, Eriksson L, Yuan K, de Jesus Perez VA, Lundberg J, Lengquist M, Botusan IR, Catrina SB, Tran PK, Hedin U, Tran-Lundmark K.
Perlecan heparan sulfate deficiency impairs pulmonary vascular development and attenuates hypoxic pulmonary hypertension.
Cardiovascular Research. 2015. May 6. In press

III. **Chang YT**, Chan CK, Eriksson I, Johnson P, Cao X, Andersson-Sjöland A, Westergren-Thorsson G, Rabinovitch M, Johansson S, Hedin U, Kjellén L, Wight TN, Tran-Lundmark K.
Versican accumulates in vascular lesions in pulmonary arterial hypertension: Regulation by hypoxia and mechanical strain.
Manuscript

IV. Tannenberg P, **Chang YT**, Folestad E, Laviña B, Genové G, Betsholtz C, Tran-Lundmark K.
Lack of PDGF-B retention ameliorates hypoxia-induced pulmonary hypertension.
Manuscript

LIST OF PUBLICATIONS NOT INCLUDED IN THESIS

I. Gotha L, Lim SY, Osherov AB, Wolff R, Qiang B, Erlich I, Nili N, Pillarisetti S, **Chang YT**, Tran PK, Tryggvason K, Hedin U, Tran-Lundmark K, Advani SL, Gilbert RE, Strauss BH.
Heparan sulfate side chains have a critical role in the inhibitory effects of perlecan on vascular smooth muscle cell response to arterial injury.
American Journal of Physiology. Heart and Circulatory Physiology. 2014;307:H337-345

II. Tseng CN, Karlöf E, **Chang YT**, Lengquist M, Rotzius P, Berggren PO, Hedin U, Eriksson EE.
Contribution of endothelial injury and inflammation in early phase to vein graft failure: The causal factors impact on the development of intimal hyperplasia in murine models.
PloS one. 2014;9:e98904

III. Tseng CN, **Chang YT**, Lengquist M, Kronqvist M, Hedin U, Eriksson EE.
Platelet adhesion on endothelium early after vein grafting mediates leukocyte recruitment and intimal hyperplasia in a murine model.
Thrombosis and Haemostasis. 2015;113:813-825

CONTENTS

LIST OF ABBREVIATIONS

BMPR	Bone morphogenetic protein receptor
CDH	Congenital diaphragmatic hernia
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
CT	Computed tomography
DEAE	Diethylaminoethyl
DS	Dermatan sulfate
EC	Endothelial cell
ECM	Extracellular matrix
ECMO	Extracorporeal membrane oxygenation
EGF	Epidermal growth factor
ERK	Extracellular signal-related kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAG	Glycosaminoglycan
HIF	Hypoxia-inducible factor
HMW	High molecular weight
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
5-HT	5-hydroxytryptophan
LMW	Low molecular weight
LV+S	Left ventricle with septum
NFAT	Nuclear factor of activated T cells
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PDE-5	Phosphodiesterase-5
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PGI_2	Prostaglandin I_2
PH	Pulmonary hypertension

PPARγ	Peroxisome proliferator-activated receptor γ
ROS	Reactive oxygen species
RV	Right ventricle
RVSP	Right ventricular systolic pressure
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMC	Smooth muscle cell
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

INTRODUCTION

CLINICAL BACKGROUND

Pulmonary hypertension (PH) is a progressive occlusive vasculopathy in the lung, associated with abnormal vasoconstriction, excessive vascular wall remodeling, inflammation and *in situ* thrombosis. Increased vascular resistance accounts for a sustained elevation of blood pressure in the pulmonary circulation, leading to increased afterload for the right ventricle and eventually to right heart failure.

The clinical manifestations of pulmonary hypertension are non-specific. The patients may have cardiopulmonary symptoms, e.g. dyspnea, chest discomfort, decreased appetite, fatigue, dizziness, and irregular heartbeat. The diagnosis of pulmonary hypertension requires a thorough physical examination and extensive tests including Doppler echocardiography and pulmonary function tests. The diagnosis should in most cases be confirmed by right heart catheterization, which when positive shows a mean pulmonary arterial pressure $\geq$ 25 mmHg at rest. Based on the clinical features and underlying etiologies, pulmonary hypertension is classified into 5 categories by the World Health Organization (WHO). Group I is pulmonary arterial hypertension (PAH), in which the pulmonary vascular pathology is pre-capillary. Group II indicates PH as a result of left heart disease. Group III PH is associated with lung diseases. Group IV PH is secondary to chronic thromboembolism and PH with unclear multifactorial mechanisms belongs to Group V. The following table (Table 1) shows the updated clinical classification as summarized at the 5th World Symposium on Pulmonary Hypertension in 2013 (Simonneau et al., 2013).

Currently established pharmacotherapy consists of prostacyclin analogues (e.g. epoprostenol, iloprost, and treprostinil), endothelin receptor antagonists (e.g. bosentan and ambrisentan), and phosphodiesterase-5 (PDE-5) inhibitors (e.g. sildenafil and tadalafil). Prostanoids exert many effects in the pulmonary vessels including vasodilatation, inhibition of proliferation and inflammation, and suppression of platelet aggregation (Clapp and Gurung, 2015). Endothelin receptor antagonists not only act as vasodilators, but also inhibit proliferation of smooth muscle cells and fibroblasts (reviewed in Humbert et al., 2014). The PDE-5 inhibitors are potent vasodilators which reduce vascular tone through the nitric oxide pathway.

In general, pulmonary hypertension is a rare but severe cardiopulmonary disorder (George et al., 2014). The above mentioned treatments mainly address endothelial dysfunction and the vasodilatory effects are the most prominent. Pulmonary hemodynamic parameters are often improved with current therapy and survival has improved to some extent, but far from enough. PH remains incurable, and the long-term prognosis is still poor (Ling et al., 2012, Benza et al., 2012, George et al., 2014). More investigations are needed to explore new therapeutic targets to improve quality of life as well as long-term outcome.

Table 1. Classification of Pulmonary Hypertension

1.	**Pulmonary arterial hypertension**
1.1	Idiopathic PAH
1.2	Heritable PAH: (i)BMPR2, (ii)ALK-1, ENG, SMAD9, CAV1, KCNK3, (iii)unknown
1.3	Drug and toxin induced
1.4	Associated with:
1.4.1	Connective tissue disease
1.4.2	HIV infection
1.4.3	Portal hypertension
1.4.4	Congenital heart diseases
1.4.5	Schistosomiasis
1'	Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
1''	Persistent pulmonary hypertension of the newborn (PPHN)
2.	**Pulmonary hypertension due to left heart disease**
2.1	Left ventricular systolic dysfunction
2.2	Left ventricular diastolic dysfunction
2.3	Valvular disease
2.4	Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3.	**Pulmonary hypertension due to lung diseases and/or hypoxia**
3.1	Chronic obstructive pulmonary disease
3.2	Interstitial lung disease
3.3	Other pulmonary diseases with mixed restrictive and obstructive pattern
3.4	Sleep-disordered breathing
3.5	Alveolar hypoventilation disorders
3.6	Chronic exposure to high altitude
3.7	Development lung diseases
4.	**Chronic thromboembolic pulmonary hypertension**
5.	**Pulmonary hypertension with unclear multifactorial mechanisms**
5.1	Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
5.2	Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
5.3	Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
5.4	Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Reprinted with permission (Simonneau et al., 2013).

PATHOBIOLOGY

The histopathological findings in PH consists of intimal hyperplasia, medial hypertrophy (thickening), distal extension of smooth muscle cells into normally non-muscular intra-acinar vessels, adventitial proliferation and fibrosis, occlusion of small arteries/plexiform lesions, thrombosis *in situ*, and infiltration of inflammatory cells and progenitor cells (Figure 1). All cell types of the pulmonary vessel wall participate in the process of vascular remodeling (Tuder et al., 2007, Tuder et al., 2013, Yeager et al., 2011, Jonigk et al., 2011).

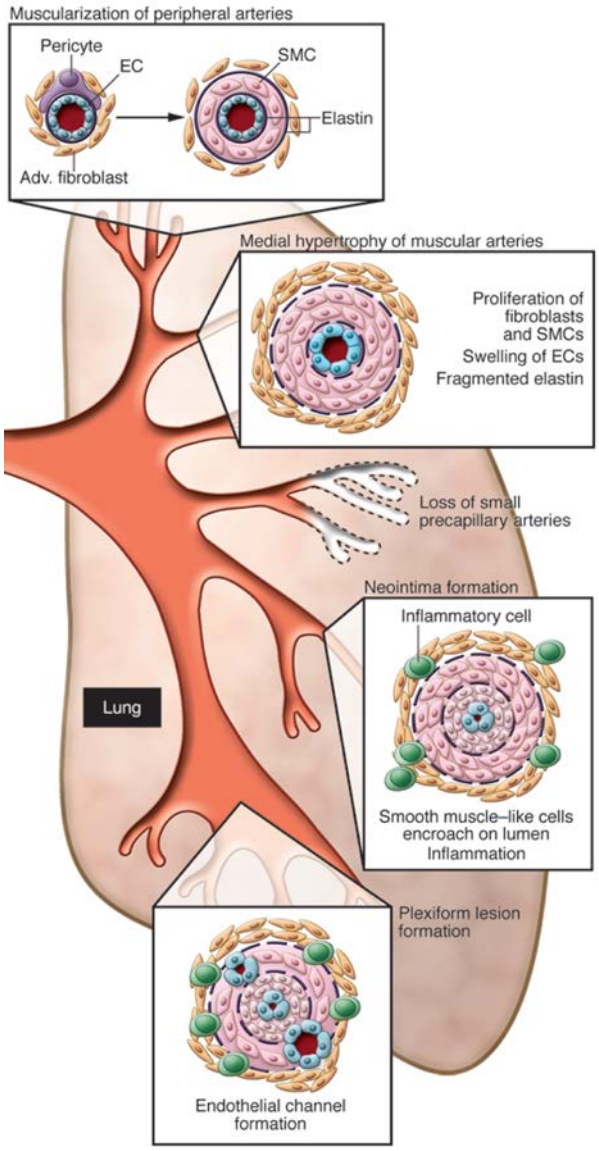


Figure 1. *Schematic illustration of vascular remodeling in PH. The pathological findings include abnormal muscularization of distal precapillary arterioles, medial wall thickening of large muscular arteries, neointima formation, loss of precapillary arterioles, and plexiform lesion formation. Reprinted with permission (Rabinovitch, 2012).*

Pulmonary artery

The pulmonary circulation is a high-flow, low-pressure, low-resistance system that carries deoxygenated blood from the right ventricle to the lungs for gas exchange. Normally, the main pulmonary artery pressure is around 20 mmHg, with systolic pressure 15-30 mHg and diastolic pressure 4-12 mmHg, as measured by right heart catheterization (Taichman et al., 2015).

In the development of the cardiovascular system the main pulmonary artery originates from the 6th pharyngeal arch at 3rd-5th week of human embryonic life. Following branching into right and left pulmonary arteries, the vessels merge with the lung buds. The proximal

pulmonary vascular branching then develops in parallel with airway branching. The distal pulmonary arteries originate from *de novo* vasculogenesis and angiogenesis within the lung parenchyma at a later developmental stage (16^{th} -26^{th} week) (Jones and Capen, 2011).

The proximal pulmonary arteries (conduit arteries) contain several layers of elastic laminae. The transitional and segmental pulmonary arteries are typically muscular arteries where smooth muscle cells (SMCs) lie between the internal elastic lamina and the external elastic lamina. The distal pulmonary arterioles are usually partially muscular or non-muscular.

Endothelial cells

Endothelial cells (ECs) form the innermost layer of the vascular wall and play important roles in maintenance of selective permeability, control of vascular tone, recruitment of inflammatory cells and regulation of thrombosis and hemostasis. Endothelial dysfunction contributes to reduced bioavailability of nitric oxide and impaired EC-dependent vasodilatation, as well as endothelial inflammatory activation and pro-thrombotic activity (Figure 2).

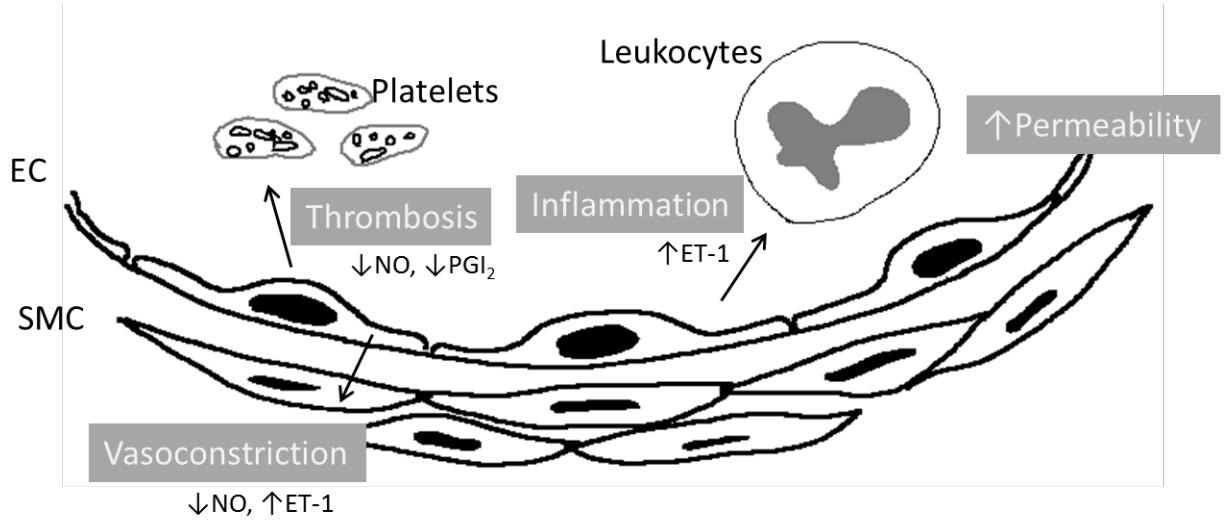


Figure 2. *Endothelial dysfunction includes impairment of EC-dependent vasodilatation, increased permeability, and activation of inflammation and thrombosis.*

In physiological conditions, ECs synthesize and release nitric oxide and prostacyclin (also called prostaglandin I_2 or PGI_2), both potent vasodilators and inhibitors of platelet aggregation. In response to environmental changes, like hypoxia or increased pressure/wall stress, ECs produce endothelins (mainly endothelin-1) to stimulate smooth muscle

contraction (Hunt et al., 2002). Endothelin-1 also recruits leukocytes (Teder and Noble, 2000, Bhavsar et al., 2008) and transactivates growth factor receptor signaling associated with smooth muscle cell proliferation (reviewed in Sandoval et al., 2014). In PH, the balance between endogenous vasodilators (e.g. decreased nitric oxide and prostacyclin) and vasoconstrictors (e.g. increased endothelin-1) is disrupted, resulting in excessive vasoconstriction and vascular remodeling (reviewed in Budhiraja et al., 2004).

Smooth muscle cells

SMCs are found in the medial layer of the vessel wall. Studies of SMCs *in vitro*, as well as in arterial tissues, support the hypothesis that the vascular SMC population is not uniform. Instead, SMCs are functionally and phenotypically heterogeneous in both systemic arteries and pulmonary arteries (Frid et al., 1994, Benzakour et al., 1996, Frid et al., 1997). In normal adult blood vessels, SMCs are in a differentiated state and have a contractile phenotype. In response to injury, SMCs phenotypically switch to a synthetic state, in which their ability to migrate, proliferate, and produce extracellular matrix is increased (Hedin et al., 2004). SMCs regulate vascular tone by vasoconstriction or relaxation. Pulmonary artery smooth muscle cell (PASMC) hypertrophy, proliferation, migration, and resistance to apoptosis contribute to vascular remodeling and play an important role in PH (Figure 3).

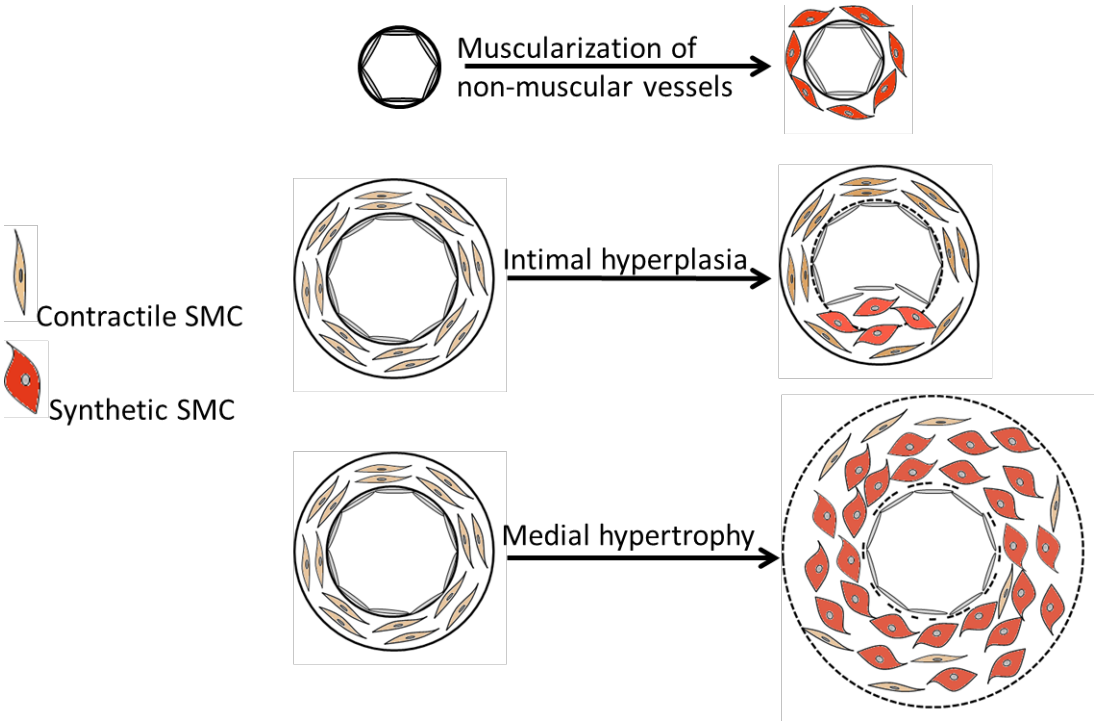


Figure 3*. The schematic diagram shows the role of SMC in vascular remodeling.*

K^+ channels

K^+ channels are the most widely distributed ion channels on cell membranes. There are four classes of K^+ channels; voltage-gated K^+ channels, Ca^{2+}-activated K^+ channels, tandem pore domain K^+ channels, and inward rectifier K^+ channels. Certain voltage-gated K^+ channels, e.g. $K_V1.2$, K_V 1.5, K_V 2.1, K_V 3.1, are oxygen-sensitive and regulated by reactive oxygen species (ROS) (reviewed in Moudgil et al., 2006). In PASMC, acute hypoxia inhibits $K_V1.5$, which in turn depolarizes the cell membrane followed by opening of voltage-gated Ca^{2+} channels (Archer et al., 1998). The resulting increase in intracellular Ca^{2+} concentration initiates hypoxic pulmonary vasoconstriction. Notably, the $K_V1.5$ protein is more abundant in distal resistance pulmonary arteries compared to proximal conduit pulmonary arteries (Archer et al., 2004). This may in part explain why hypoxic pulmonary vasoconstriction predominantly occurs in distal intra-acinar pulmonary arteries.

Free cytosolic Ca2+

Intracellular calcium ion concentration is regulated by calcium influx through calcium ion channels and intracellular release from the sarcoplasmic reticulum. The free cytosolic Ca^{2+} level is the key determinant of SMC contraction. In addition, increased intracellular free calcium is essential for growth factor-induced SMC proliferation (Mogami and Kojima, 1993, Magnier-Gaubil et al., 1996).

Serotonin

Serotonin, or 5-hydroxytryptophan (5-HT), is a monoamine neurotransmitter derived from the amino acid tryptophan. Serotonin can be found in the gastrointestinal tract, the central nervous system, platelets, and also in ECs. Several studies have demonstrated that serotonin mediates vasoconstriction and PASMC proliferation (Kahn et al., 1992, Pitt et al., 1994, Lee et al., 1994). Overproduction of serotonin in pulmonary ECs derived from patients with PH has also been shown to increase PASMC growth (Eddahibi et al., 2006). Furthermore, the serotonin transporter responsible for internalization of serotonin is of importance for PASMC proliferation in PH lungs (Marcos et al., 2004).

Growth factors

Transforming growth factor (TGF) and bone morphogenetic protein receptor (BMPR)

The TGF-β superfamily is comprised of a large family of cytokine growth factors including TGF-β proteins, bone morphogenetic proteins, activins, and other regulatory proteins. TGF-β not only controls cell cycle progression, cell differentiation and ECM protein secretion, but

also has a crucial role in cell re-programming and epithelial-mesenchymal transition (Xie et al., 2013, reviewed in Massague, 2012).

BMPR2 is one of the receptors for growth factors in the TGF-β superfamily. BMPR2 loss of function mutations have been shown to be associated with hereditary PAH and some sporadic idiopathic PAH (International et al., 2000, Deng et al., 2000). Several studies have revealed that BMPR2 signaling promotes EC survival while SMC proliferation is inhibited (de Jesus Perez et al., 2009, Hansmann et al., 2008). However, most family members who carry BMPR2 mutations do no develop familial PAH (Hamid et al., 2009), suggesting that other factors affect disease penetrance.

Platelet-derived growth factors (PDGFs)

PDGF was first identified as a platelet-dependent serum factor for SMC and fibroblast proliferation (Kohler and Lipton, 1974, Ross et al., 1974). So far the PDGF family comprises four polypeptides (PDGF-A, PDGF-B, PDGF-C and PDGF-D) that can form dimeric isoforms. *In vivo,* PDGFs form active disulfide-linked homodimers that bind PDGF receptor-α or PDGF receptor-β (PDGFR-β) (Figure 4). One heterodimer, PDGF-AB, has been found in human platelets (Hammacher et al., 1988). However, the role of PDGF-AB remains unclear.

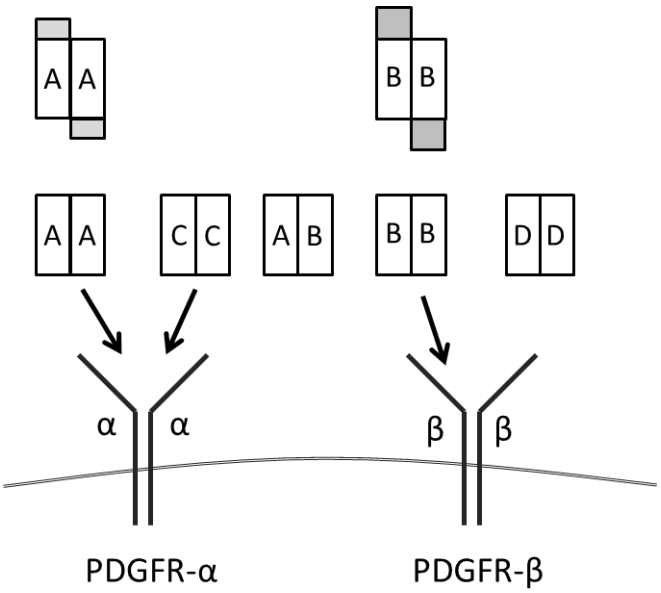


Figure 4. *The illustration shows the PDGF-PDGFR interaction in vivo. Gray rectangles indicate retention motifs comprising basic amino acids residues (adapted from Andrae et al., 2008).*

PDGFs act as mitogens and chemoattractants for cells of mesenchymal origin. In particular, PDGF-B is a strong mitogen for SMCs. Often more potent than fibroblast growth factor-2. PDGF-B is especially important in the vasculature and plays a critical role in cell proliferation, migration, differentiation and angiogenesis (see reviews, Hughes et al., 1996, Heldin and Westermark, 1999, Hoch and Soriano, 2003, Andrae et al., 2008).

In the angiogenic process, PDGF-B/ PDGFR-β interaction is crucial for recruitment of mural cells, pericytes and SMCs, to cover nascent endothelial sprouts (Lindahl et al., 1997, Enge et al., 2002). PDGF-B released from vascular ECs promote proliferation of PDGFR-β expressing pericytes and SMCs. PDGF-B also acts as chemoattractant to direct the migration of pericytes and SMCs along newly formed endothelial sprouts (Hellstrom et al., 1999).

It has been shown that the carboxy-terminus of PDGF-B has a high proportion of basic amino acid residues, which is important for cell membrane retention properties (Ostman et al., 1991, LaRochelle et al., 1991). Extracellular proteolytic cleavage of those "retention motifs" is thought to determine the release of PDGF-B from cell association or storage in the extracellular matrix. The mitogenic activity is retained after cleavage. The binding of PDGF retention motifs to negatively charged proteoglycans may be important for chemotactic gradients guiding recruitment of mural cells. Defective pericyte attachment in microvessels of PDGF-B retention motif knockout mice (*Pdgfb* $^{ret/ret}$) supports this hypothesis (Lindblom et al., 2003).

Aberrant PDGF regulation and signaling has been demonstrated in many studies of PH. Upregulation of PDGF transcripts was reported in a rodent model of hypoxic PH (Katayose et al., 1993) and increased PDGF receptor expression was observed in a lamb model with perinatal PH induced by ligation of the ductus arteriosus (Balasubramaniam et al., 2003). PDGF inhibition by imatinib, a tyrosine kinase inhibitor, successfully reverses PH in both chronic hypoxic mice and monocrotaline-treated rats (Schermuly et al., 2005). In patients with idiopathic PAH, overproduction of PDGF and PDGFR-β has been found in small pulmonary arteries, mainly localized to PASMC and ECs with perivascular inflammation (Perros et al., 2008). Imatinib treatment of patients with PAH reached phase III clinical trials and was shown to improve hemodynamics and right heart function in patients with severe PAH. However, imatinib has not been approved for use in PH due to severe side effects such as subdural hematomas (Hoeper et al., 2013, Shah et al., 2015).

Fibroblast growth factors (FGFs)

Many decades ago, FGFs were identified as mitogens and angiogenic factors. Currently, the FGF family consists of 18 members (FGF1-FGF10, and FGF16-FGF23). FGF11-FGF14 do not activate FGF receptors and are therefore not considered as part of the FGF family (Beenken and Mohammadi, 2009). By activating the FGF receptor tyrosine kinases (FGFR1-FGFR4), FGFs are known to have a vast range of biological functions both in development and diseases.

FGF-2, or basic fibroblast growth factor, has been widely used as a mitogen and chemoattractant for ECs and SMCs *in vitro*. However, the physiological function of FGF-2 has not been fully elucidated. FGF-2 can be translated into different isoforms from a single mRNA. In rodents, 3 isoforms with molecular weight of 18 kDa, 22 kDa, and 25 kDa have been identified (Shing et al., 1984, Presta et al., 1988, Moscatelli et al., 1987), while 5 isoforms have been identified in humans (Abraham et al., 1986, Florkiewicz and Sommer, 1989, Arnaud et al., 1999).

The 18 kDa FGF-2, or low molecular weight (LMW)-FGF-2 is translated from a conventional AUG start codon, whereas others (high molecular weight (HMW)-FGF-2) are initiated by alternative upstream CUG codons (Prats et al., 1989) (Figure 5). LMW-FGF-2 is released into the extracellular space, but can also be found in the nucleoli. HMW-FGF-2 predominantly localizes in the nucleus. However, a recent report showed that HMW-FGF-2 can be released extracellularly (Santiago et al., 2011).

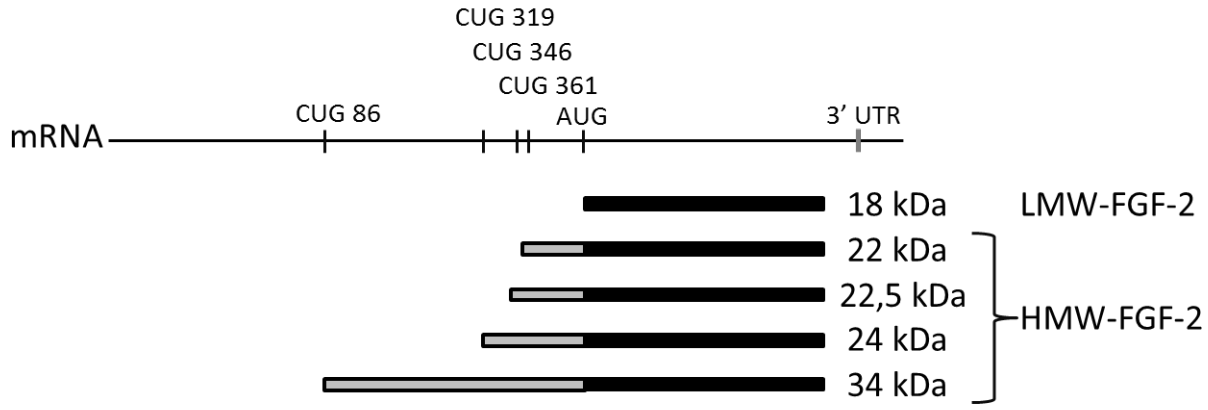


Figure 5. *FGF-2 isoforms*

In vascular biology, compelling evidence has revealed that LMW-FGF-2 is essential for endothelial sprouting in angiogenesis (reviewed in Lieu et al., 2011). On the other hand, HMW-FGF-2 contributes to cardiac hypertrophy both *in vitro* and *in vivo* (Jiang et al., 2007, Santiago et al., 2010).

Notably, overproduction of FGF-2 has been shown in patients with PH (Benisty et al., 2004) as well as in an animal model (Wedgwood et al., 2007). Furthermore, it has been demonstrated that endothelial-derived FGF-2 contributes to SMC proliferation in humans and rats, and the use of either FGF-2 siRNA or an FGFR inhibitor inhibits vascular remodeling and nearly reverses PH development in monocrotaline-treated rats (Izikki et al., 2009). Collectively, the data suggest that FGF-2 may become a therapeutic target for treatment of PH.

In addition to TGF-β, PDGF-B, and FGF-2, other growth factors like vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), take part in the vascular

remodeling in PH (Partovian et al., 1998, Geiger et al., 2000, Farkas et al., 2009, Dahal et al., 2010), but their roles will not be described in detail in this thesis.

Transcription factors

Hypoxia-inducible factors (HIFs)

HIFs are transcription factors that function as key sensors for cellular oxygenation. Through binding to the hypoxia-responsive elements in the promotor region of hundreds of genes and activation of gene transcription, multiple biochemical events are triggered and cells are able to adapt to the low oxygen level for survival (reviewed in Greer et al., 2012).

HIF-1 is composed of an oxygen-sensitive α subunit and a constitutively expressed β subunit. In normoxia, the α subunit is rapidly degraded while under hypoxia the degradation is inhibited, which stabilizes the dimerization of HIF-1α with HIF-1β (Wang et al., 1995, Wang and Semenza, 1995). HIF-1α is an essential element for the physiological response to chronic hypoxia and has been shown to contribute to hypoxia-induced PH (Yu et al., 1999, Wang et al., 2006). HIF-1α can induce endothelin-1 expression in ECs (Hu et al., 1998) and endothelin-1 will also increase HIF-1α synthesis in SMCs (Pisarcik et al., 2013). A recent study showed reduced hypoxia-induced pulmonary vascular remodeling in SMC-specific HIF-1α inducible knockout mice (Ball et al., 2014), which further supports a central role of HIF-1α in the development of PH.

Peroxisome proliferator-activated receptor γ (PPAR γ)

PPAR γ is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. PPAR γ has a DNA-binding domain and a ligand-binding domain and it is expressed in ECs, SMCs, cardiomyocytes, and macrophages. In normal conditions, PPAR γ is widely expressed in various cell types in the lung. In patients with PAH, PPAR γ is decreased in plexiform lesions, contributing to apoptosis-resistance properties of ECs (Ameshima et al., 2003). Reduction of PPAR γ expression has been found in animal models of PH, and PPAR γ activation by use of PPAR γ ligands such as pioglitazone or troglitazone have been shown to reduce PH in animal models. Recent investigations suggest that PPAR γ takes part in many pathways implicated in PH pathobiology (reviewed in Green et al., 2011). PPAR γ activation can reduce vasoconstriction and vascular remodeling. In addition, it has anti-inflammatory and anti-thrombotic effects. However, since the clinical use of PPAR γ ligands for other purposes has revealed increased risk of myocardial infarction and heart failure (Nissen and Wolski, 2007, Lincoff et al., 2007) clinical use in PH is not considered currently.

Wnt/β-catenin

The canonical Wnt/ β-catenin pathway regulates cell proliferation and differentiation and plays important roles in embryonic development and tissue homeostasis. It has been shown

that Wnt7b, expressed in lung epithelium, regulates mesenchymal differentiation essential for PASMC development in the mouse lung. Wnt7b/ β-catenin promote proliferation of PASMC precursors via activation of tenascin C, which is essential for PDGF receptor expression (Cohen et al., 2009).

Nuclear factor of activated T cells (NFAT)

NFAT is a $Ca^{2+/}$calcineurin-sensitive transcription factor. It has been demonstrated that activated NAFTc2 in PASMC from patients with IPAH contributes to decreased K^+ channel $K_V1.5$ expression, increased intracellular free Ca^{2+} and K^+ concentrations and PASMC contraction as well as proliferation (Bonnet et al., 2007).

Pericytes

Pericytes, also known as Rouget cells, are mural cells which have *"peg-socket contacts"* with the endothelium in microvessels (reviewed in Armulik et al., 2005). Pericytes are present in precapillary arterioles, capillaries, and postcapillary venules and share the basement membrane with ECs. Even though the identity of pericytes is difficult to define, this cell type can be distinguished from SMCs based on location, morphology, and specific protein expression markers.

Pericytes have multiple cytoplasmic processes which cover the abluminal surface of the endothelium (Figure 6), and pericytes have been shown to be of importance in blood vessel formation, blood flow regulation, maintenance of capillary integrity and permeability, and vascular remodeling (reviewed in Armulik et al., 2011).

In the human lung, pericytes have been shown to be present in alveolar capillaries (transmission electron microscopic images in Weibel, 1974), where the pericytes have few and small processes. Recently, pericytes have been shown to be associated with development of pulmonary fibrosis (Hung et al., 2013) and pulmonary hypertension (Ricard et al., 2014, Yuan et al., 2015). However, the function of pericytes in the lung and the role in pulmonary vascular remodeling has not been fully determined.

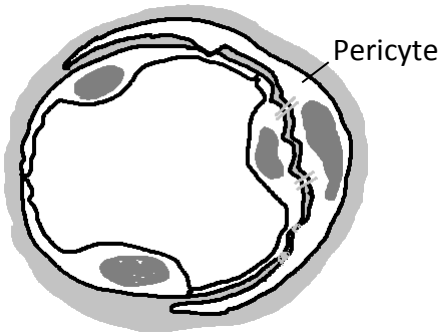


Figure 6. *The illustration shows a pericyte which encircles the capillary endothelium and shares the basement membrane with the endothelium.*

Other vascular cell types

The sections above mainly describe the role of ECs and mural cells in pulmonary vascular remodeling. It should however be noted that other cell types like fibroblasts, fibrocytes, inflammatory cells, and different types of progenitor cells also participate in vascular remodeling of PH and the role of these cells have been nicely reviewed elsewhere (Stenmark et al., 2012, Savai et al., 2012, Yeager et al., 2011).

Extracellular matrix

The extracellular matrix (ECM) of the vascular wall is the non-cellular component comprised of interstitial matrix and basement membranes. The vascular cells synthesize and release the ECM components, including fibrous proteins (e.g. elastin and collagen), glycoproteins (e.g. laminins), glycosaminoglycans, and proteoglycans. The ECM makes up a structural scaffold, but it also has versatile elastic properties of importance to protect the vessel against changes in mechanical forces. In addition, the ECM interacts with various chemokines, cytokines, growth factors, and cell-surface receptors to regulate cell adhesion, migration, differentiation and growth (reviewed in Mouw et al., 2014).

The basement membrane is a specialized form of ECM which lies underneath the endothelium and separates the cell monolayer from the underlying connective tissue. Collagen IV, laminin, nidogen/entactin and perlecan are the major components of the basement membrane (LeBleu et al., 2007). SMCs in the vessel media are also surrounded by a basement membrane (Risau and Lemmon, 1988), which has been shown to play an essential role in the control of SMC phenotype and growth (Hedin et al., 1999).

Elastin

Elastin fibers form extensible elastic lamellae, essential for the elastic properties of large conduit arteries. The elastin precursor, tropoelastin, is a hydrophobic protein, which self-associates by covalent cross-linking immediately after secretion to form elastic fibers with long-lasting capacity. In healthy vessels, the estimated half-life of elastin is 74 years (Shapiro et al., 1991). Elastin peptides are degradation products of elastic fibers and have been shown to be of importance in diseases such as atherosclerosis, diabetes, and aortic aneurysms (reviewed in Qin, 2015). It has also been shown that elastin degradation is associated with PH development (Todorovich-Hunter et al., 1988, Perkett et al., 1991). Loss of elastin contributes to increased stiffness of proximal pulmonary arteries (Lammers et al., 2008). Notably, increased activity of neutrophil elastase, the elastin degradation enzyme produced from neutrophils and PASMCs, has been linked to neointimal lesions in patients with PAH (Kim et al., 2011). It is likely that degradation of elastin in the ECM facilitates SMC proliferation and migration.

Collagen

Collagens are the most abundant fibrous proteins in the body. Collagen IV is one of the principle components of basement membranes in the vascular wall. More than 20 years ago, it was observed that collagen synthesis is increased in different vascular lesions of PH (Botney et al., 1993). Recently, it was reported that increased procollagen levels are positively correlated with severity of disease in patients with PH (Safdar et al., 2014).

Fibronectin

Fibronectin is a large glycoprotein in the ECM. In addition to binding to integrins, fibronectin can interact with several ECM components such as collagen, fibrin, tenascin C and heparan sulfate proteoglycans. Increased fibronectin has been shown both in animal models of PH and in patients with PH (Durmowicz et al., 1994, Jones et al., 1997). In addition, fibronectin can be serotonylated, which means that serotonin is attached to glutamine residues of fibronectin. Serotonylation has been show to promote pulmonary artery SMC proliferation and migration (Watts et al., 2009, Wei et al., 2012).

Tenascin C

Tenascin C is a glycoprotein in the ECM. Tenascin C can interact with fibronectin and inhibit fibronectin fibrillogenesis (Chung et al., 1995, To and Midwood, 2011). Increased tenascin C in neointima and obliterative lesions has been reported in patients with PH (Jones et al., 1997). It has also been shown that loss of BMPR2 induces tenascin C production, which in turn promotes SMC proliferation (Ihida-Stansbury et al., 2006).

Hyaluronan

Hyaluronan is a large non-sulfated glycosaminoglycan synthesized on the plasma membrane and deposited in the ECM. Hyaluronan accumulates in plexiform lesions in patients with PAH (Aytekin et al., 2008), where hyaluronan binds to heavy chains of the plasma protein, inter-alpha-trypsin inhibitor, and forms a cable-like structure to faciliate leukocyte adhesion (Lauer et al., 2014).

Inflammation

A role of inflammation in PH was initially identified 20 years ago (Tuder et al., 1994). Since then, a large numbers of investigations, using both animal models and human material, have provided convincing evidence that inflammation plays a crucial role in PH development (see reviews, Price et al., 2012, Huertas et al., 2014, Rabinovitch et al., 2014). Dysregulation of T cells (mostly aberrant Treg function) and macrophage recruitment and activation are common

characteristics in vascular lesions of patients with PAH and in animal models of PH. Increased levels of autoantibodies and increased number of circulating dendritic cells have also been reported in several studies, but to a lesser extent. On the other hand, increased cytokines and chemokines, like IL-1-β, IL-6, IL-8, interferon-γ, and tumor necrosis factor-α, have been observed in patients with PAH and may be related to disease severity.

120 years of research

The earliest report of vascular lesions associated with PH can be traced back more than 120 years. In 1891 a German physician, Ernst von Romberg, described a condition which he called "pulmonary vascular sclerosis" (Romberg, 1891). Since then, thousands of studies have been conducted to delineate the disease mechanisms in order to develop effective treatments. Endothelial dysfunction, excessive vascular remodeling, inflammation and *in situ* thrombosis are the major elements in the pathobiology of PH, and many cell types in the pulmonary vasculature take part in the remodeling process. The illustration below (Figure 7) shows the current understanding of the disease mechanisms.

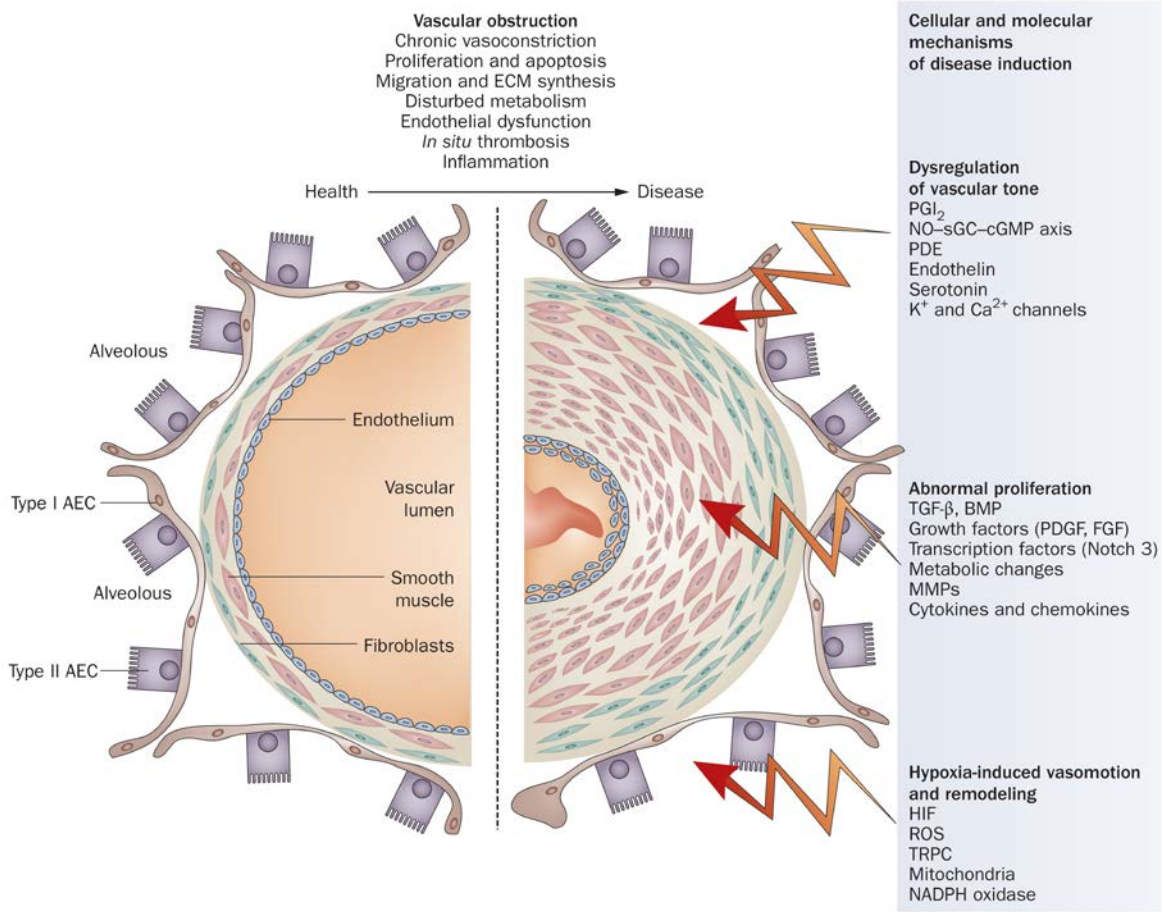


Figure 7. *The pathogenic mechanisms of PH. Reprinted with permission (Schermuly et al., 2011).*

PROTEOGLYCANS

Proteoglycans are complex molecules composed of a core protein with covalently bound linear polysaccharides called glycosaminoglycans (GAGs) (Iozzo and Murdoch, 1996). The synthesis of core proteins takes place in the endoplasmic reticulum, followed by GAG synthesis and post-translational modifications in the Golgi apparatus (Prydz and Dalen, 2000). After transport to the cell surface, the proteoglycans are either deposited in the ECM or retained at the cell surface.

The GAGs of proteoglycans are polymerized and modified by glycosyltransferases, sulfotransferases and epimerases. Based on the structure of repeating disaccharide units, the sulfated GAGs can be heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), or keratan sulfate. Heparin is a highly sulfated variant of HS. The basic disaccharide unit in HS is glucuronic acid linked to an *N*-acetylglucosamine residue, whereas CS comprises glucuronic acid and *N*-acetylgalactosamine (Figure 8). DS is similar to CS but contains in addition to glucuronic acid also its 5'-epimer iduronic acid. Keratan sulfate is a sulfated polymer of N-acetyl-lactosamine.

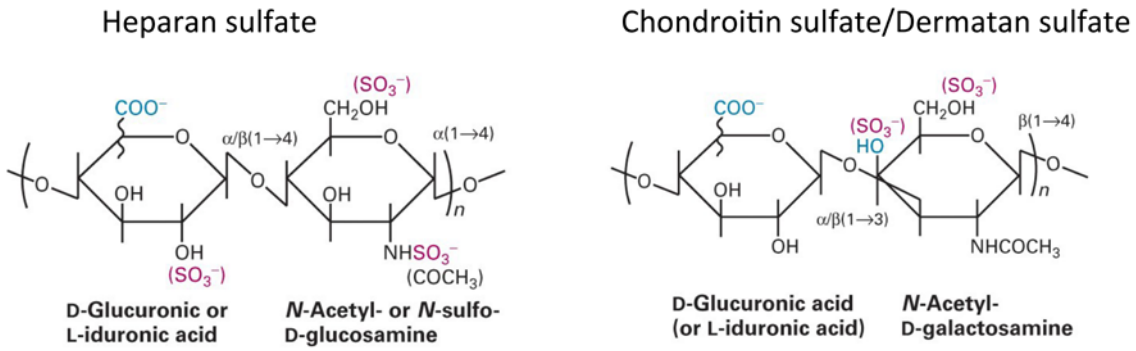


Figure 8. Disaccharide units in GAGs.

HS is a complex and heterogeneous structure of unbranched polysaccharide with various sulfation patterns and chain lengths. The synthesis of HS chains is initiated by the formation of a tetrasaccharide linkage region on selected serine residues of core proteins, followed by transfer of the first *N*-acetylglucosamine residue to the linkage region and stepwise addition of glucuronic acid and *N*-acetylglucosamine. The extended chains are further modified by sulfotransferases and an epimerase (Kreuger and Kjellen, 2012).

HS chains have diverse functions, including modulation of mitogen and morphogen signaling (Jastrebova et al., 2006), clearance and retention of lipoproteins (MacArthur et al., 2007, Tran-Lundmark et al., 2008), direction of neutrophil infiltration (Wang et al., 2005), stimulation of appetite (Reizes et al., 2001), and facilitation of amyloid deposition (Scholefield et al., 2003). It has been proposed that the overall negative charge density on HS chains determines the interaction between HS and proteins (see reviews, Bishop et al., 2007, Lindahl and Kjellen, 2013).

Like HS, CS chains also have variable sulfation patterns and may possess many different biological activities. CS-protein interactions are possible through electrostatic charge, similar to HS-protein interactions. However, the function and actual mechanisms have not been as well characterized as those for HS.

In general, proteoglycans are widely expressed on the cell surface and in the ECM. An exception is serglycin, which is found in the secretory granules of mast cells and other myeloid cells. Studies over the past 40 years have revealed that proteoglycans not only take part in ECM assembly, but also play pivotal roles in cell adhesion, proliferation, migration, and differentiation during embryonic development and tissue remodeling (reviewed in Iozzo and Schaefer, 2015).

The following sections describe some important proteoglycans in the vascular wall.

Perlecan

Perlecan is the largest heparan sulfate proteoglycan (HSPG) and the major HSPG in the basement membrane. It was initially isolated from the basement membrane of the Engelbreth-Holm-Swarm sarcoma and obtained the name based on the ultrastructural feature of "beads on a string" revealed by rotary shadowing electron microscopy (Hassell et al., 1980, Paulsson et al., 1987, Yurchenco et al., 1987). Complete sequencing and protein structure characterization have demonstrated that this macromolecule (~800 kDa) contains 5 globular domains with 4391 amino acids (~470 kDa) and 3 HS chains attached to the N-terminus and one chain to the C-terminus (Figure 9) (Noonan et al., 1991, Murdoch et al., 1992, Cohen et al., 1993).

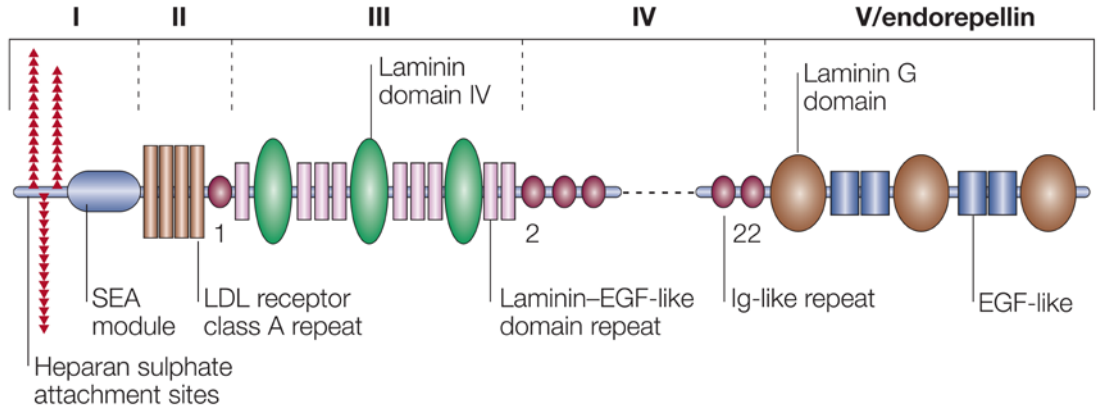


Figure 9. *Schematic illustration of perlecan. Domain I contains three Ser-Gly-Asp triplets that are HS binding sites. The additional HS binding site in domain V is not shown in this picture. Reprinted with permission (Iozzo, 2005).*

In blood vessels, perlecan is synthesized by ECs and SMCs, and deposited in basement membranes. Perlecan is not only part of the ECM scaffold, but also acts as a modulator for growth factors. The heparan sulfate (HS) chains linked to domain I are able to bind various growth factors (e.g. FGF, VEGF, PDGF) (Ornitz, 2000, Knox et al., 2002, Zoeller et al., 2009, Ishijima et al., 2012) and function as modulator by presenting the growth factors to the cognate signaling receptors or by sequestering growth factors for storage (Figure 10). In addition, the negative charge of HS takes part in maintenance of gradients for mitogens and morphogens in the extracellular space (Abramsson et al., 2007).

In vascular biology, perlecan HS has been shown to inhibit SMC proliferation in *vitro* through hampering FGF-2 activity (Nugent et al., 1993, Forsten et al., 1997). Our group has previously shown that perlecan HS inhibits intimal hyperplasia in the carotid ligation model, possibly through sequestering FGF-2 in the ECM (Tran et al., 2004). On the other hand, perlecan HS can serve as a functional docking platform for FGF-2. A recent study showed that perlecan is upregulated in neointima lesions in the renal transplantation model in which perlecan HS promotes FGF-2 activity (Katta et al., 2013). Perlecan HS also promotes angiogenesis by formation of the perlecan/FGF-2 complex resistant to proteolysis (Saksela and Rifkin, 1990, Aviezer et al., 1994), or by maintenance of the basement membrane integrity (Stratman et al., 2009, Gustafsson et al., 2013). It has been reported that perlecan HS derived from different cell types modulate FGF signaling in different ways (Knox et al., 2002, Lord et al., 2014). The dual function of perlecan HS may imply the presence of different HS structures due to cell type specific post-translational modifications.

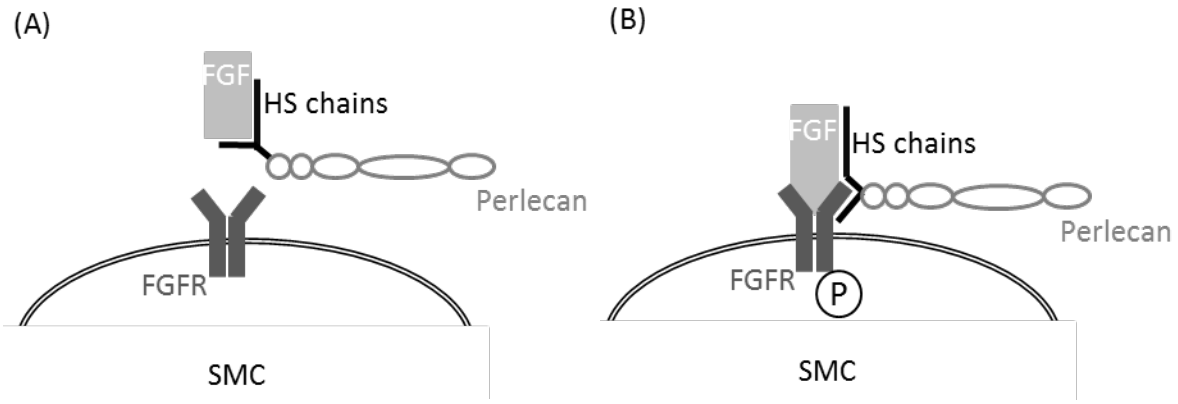


Figure 10. *The duality of perlecan HS. (A)Perlecan HS chains sequester FGFs to inhibit FGFs activity (B) Perlecan HS chains function as a docking platform for FGFs to promote signaling.*

While HS chains on the N-terminal have pro-angiogenic properties, the C-terminal of perlecan harbors anti-angiogenic properties. Endorepellin, a fragment from the C-terminal of perlecan containing three laminin-like globular domains, has been shown to inhibit

angiogenesis via binding to the α2β1 integrin and to VEGF receptor 2, which in turn suppresses HIF-1α and NFAT activity (Mongiat et al., 2003, Goyal et al., 2012). Endorepellin can be cleaved by the BMP-1/Tolloid proteases, releasing the LG3 domain with potent angiostatic activity (Gonzalez et al., 2005, Le et al., 2011). On the other hand, LG3 facilitates SMC migration and is associated with neointima formation after renal allograft transplantation (Soulez et al., 2012).

Syndecans

Syndecans are cell surface HSPGs with transmembrane domains (Figure 11). In addition to HS, CS GAG chains may also be attached to the syndecan core proteins. In vertebrates, there are four syndecan members (syndecan-1, -2, -3, and -4) expressed by distinct genes on different chromosomes. Syndecans are important regulators for cell-cell and cell-matrix interactions. In addition, syndecans like other HSPGs can modulate growth factor signaling. Syndecan-1 has been shown to inhibit intimal hyperplasia and keep SMCs in a differentiated and quiescent state (Fukai et al., 2009, Chaterji et al., 2014).

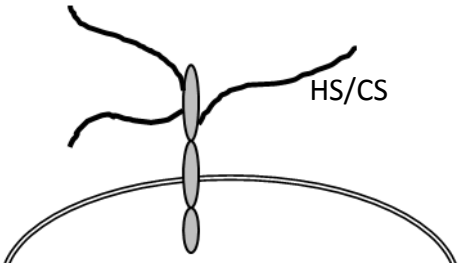


Figure 11. *Syndecan*

Glypicans

Glypicans are cell surface HSPGs that are linked to the cell membrane by a glycosylphosphatidylinositol anchor (Figure 12). In mammals, six glypicans (GPC1-GPC6) have been identified. Glypicans have been shown to modulate the activity of FGFs, Wnt, and Hedgehog (Gutierrez and Brandan, 2010, Capurro et al., 2005, Capurro et al., 2008). Glypican-3 deficient mice display developmental overgrowth and cardiac malformation, suggesting that glypican-3 is important for developmental morphogenesis (Ng et al., 2009).

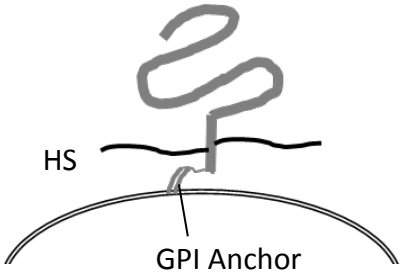


Figure 12. *Glypican*

Versican

Versican is the largest chondroitin sulfate proteoglycan (CSPG) in the ECM. The eponym, versican, comes from its versatile function and complex structure (Zimmermann and Ruoslahti, 1989). Versican exists in at least four isoforms, V0, V1, V2, and V3. The full length versican core protein (V0) has a molecular weight of around 400 kDa with 3396 amino acid residues and is composed of the G1domain at the N-terminus, the αGAG domain (encoded by exon 7) and the βGAG domain (encoded by exon 8) in the central region with CS attachment sites, and the G3 domain at the C-terminus. Alternative splicing of the GAG attachment domains generates at least 3 additional isoforms, V1 (with βGAG domain), V2 (with αGAG domain), and V3 (no GAG binding domain) (Figure 13) (Naso et al., 1994). V0 and V1 isoforms are present in the developing heart and in vascular SMCs (Yao et al., 1994), V2 is a major component of the ECM which surrounds myelinated fibers in the brain (Schmalfeldt et al., 1998).

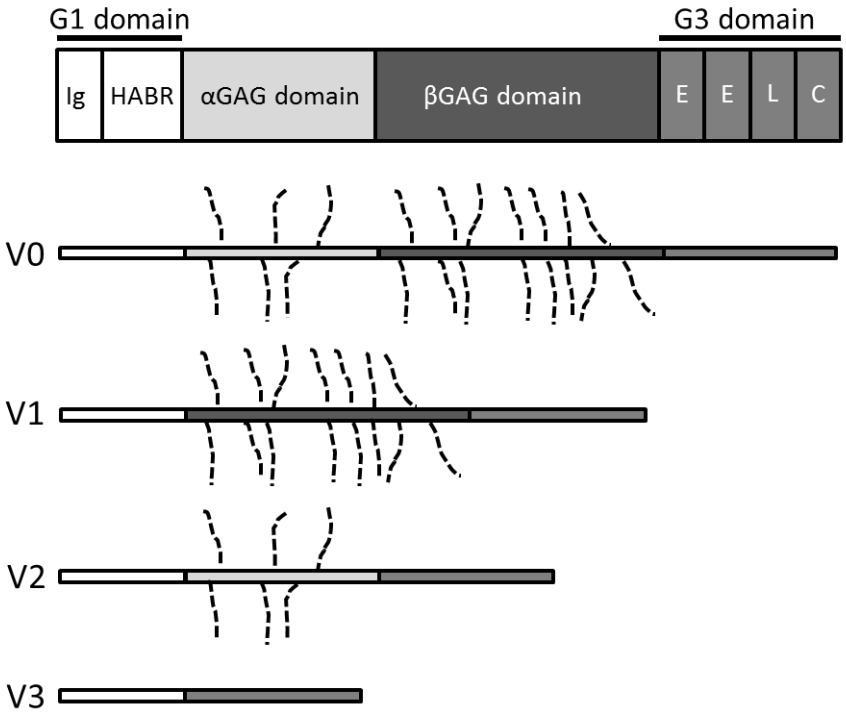


Figure 13. *Schematic model of the versican core protein and its isoforms. Versican has two globular domains, the G1 domain at the amino terminus and the G3 domain at the carboxy terminus. The splice variants vary in the GAG attachment domains (αGAG and βGAG). Ig: immunoglobulin-like domain; HABR: hyaluronan binding region; E: epidermal growth factor-like domain; L: lectin binding domain and C: complement regulatory region. Dashes represent CS chains.*

In general, versican protein levels are low in connective tissue and increase in various pathological conditions (reviewed in Theocharis, 2008). By binding to molecules like growth factors, chemokines and ECM proteins, versican takes part in diverse biological activities through regulating cell adhesion, migration, proliferation and differentiation.

A large number of studies have demonstrated that versican accumulates in vascular diseases like atherosclerosis and restenosis, where versican is essential for SMC growth, lipoprotein retention, and retention of inflammatory cytokines (reviewed in Wight and Merrilees, 2004). For example, the N-terminal of versican can bind to hyaluronan and form large aggregates in the ECM, which have been shown to increase SMC proliferation and migration as well as to retain leukocytes in the ECM (LeBaron et al., 1992, Evanko et al., 1999, Evanko et al., 2012). On the other hand, the V3 variant has been shown to inhibit SMC proliferation. In addition, versican with CS chains may directly or indirectly disturb elastin synthesis and elastic fiber formation (Merrilees et al., 2011).

Decorin

Decorin is one of the small leucine-rich proteoglycans (Figure 14). The eponym was coined based on its ability to decorate collagen fibrils near the D band (Krusius and Ruoslahti, 1986, Pringle and Dodd, 1990, Fleischmajer et al., 1991). In addition to regulating collagen fibril formation, decorin is able to control cell growth through interacting with many growth factors and growth factor receptors. For example, decorin can inhibit proliferation by inducing EGF receptor internalization and degradation (Zhu et al., 2005). It has also been shown to suppress HIF-1α and VEGFA (Neill et al., 2012).

In the lung, decorin inhibits TGF-β activity, which in turn reduces pulmonary fibrosis (Kolb et al., 2001). In vascular pathology, decorin has been shown to be deposited prominently in the ECM of the fibrous cap of atherosclerotic plaque, where there is collagen accumulation (Radhakrishnamurthy et al., 1998). Cyclic mechanical strain on aortic SMC reduces decorin production while versican is upregulated (Lee et al., 2001). Interestingly, it has been demonstrated that decorin gene delivery reduces cardiac fibrosis in hypertensive rats (Yan et al., 2009) and inhibits neointima formation in an *ex vivo* model of venous grafting (Ranjzad et al., 2009).

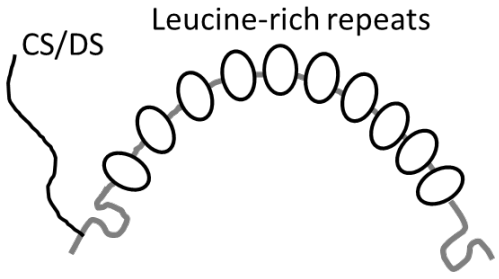


Figure 14. *Decorin*

Biglycan

Biglycan is also one of the small leucine-rich proteoglycans in the ECM. It is structurally similar to decorin with > 65% overall homology (Figure 15). In atherosclerosis, biglycan prominently accumulates in the intima and co-localizes with versican (Lin et al., 1996). Biglycan has been shown to promote SMC proliferation and migration (Shimizu-Hirota et al., 2004). Furthermore, the GAG chains of biglycan take part in lipid retention (O'Brien et al., 2004). Recently, increased atherosclerosis development was reported in mice with SMC-specific biglycan overexpression (Thompson et al., 2014). However, biglycan-deficient mice are not protected against atherosclerosis. Instead, the mice are predisposed to angiotensin II-induced aortic aneurysm formation, concomitant with increased vascular perlecan content (Tang et al., 2014).

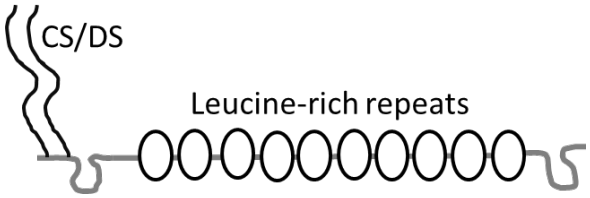


Figure 15. *Biglycan. The eponym was coined because this proteoglycan contain two GAG chains.*

The two largest proteoglycans in the extracellular matrix, perlecan and versican, are the main focus of this thesis (Paper II and III). The role of perlecan and versican in vascular remodeling in PH are discussed in the “Results and Discussion” section.

AIM OF THE STUDY

As mentioned in the “Introduction” section, current treatments for PH with endothelin receptor antagonists, prostacyclin analogues, and PDE-5 inhibitors have limited effects on the long-term outcome and the 5-year mortality rates for patients with PH are still high, ranging from 40%-80% (Thenappan et al., 2012). More research is therefore needed to find better diagnostic tools and better pharmacological treatments, especially targeting excessive vascular cell proliferation. Deeper insight into the vascular remodeling of PH is required to design new pharmacological therapies.

The general aim of this thesis was to study the regulation of SMC growth and the role of ECM proteoglycans in vascular remodeling in PH.

The specific aims were:

Paper I: To investigate the effect of imatinib on pulmonary vascular remodeling associated with congenital diaphragmatic hernia.

Paper II: To study the role of basement membrane perlecan HS in the vascular remodeling process of hypoxia-induced PH.

Paper III: To explore the role of versican, the major CSPG in the vascular ECM, in vascular lesions of PAH.

Paper IV: To examine the role of the PDGF-B retention motif in pulmonary vascular remodeling in hypoxia-induced PH.

MATERIALS AND METHODS

PRECLINICAL MODELS OF PULMONARY HYPERTENSION

PH is a devastating vascular disorder. In order to investigate the pathobiology and design effective pharmacological therapies, several animal models of PH have been established during the past decades. The use of animal models allows us to study the disease progress at different time points. This approach provides valuable insight into pathogenic mechanisms and also benefits innovation of pharmacological agents.

Chronic hypoxia

Chronic hypoxia exposure (10% O_2) can be used to induce vascular remodeling in many different species (e.g. mice, rats, and calves) and it is the most commonly used model, while monocrotaline injection is widely used in rats to induce PH. The advantage of the chronic hypoxia model is the simplicity and reproducibility. The vascular changes seen comprise distal muscularization of normally nonmuscularized intra-acinar arterioles, thickening of the tunica media, adventitial fibrosis, and perivascular inflammatory infiltration (Rabinovitch et al., 1979, Stenmark et al., 2006). In addition, ECM remodeling and stiffening of proximal conduit pulmonary arteries have been observed (Lammers et al., 2008, Ooi et al., 2010). In general, the vascular remodeling seen with hypoxia, especially in mice, is not severe and there is no neointima formation and no plexiform lesions (Stenmark et al., 2006). Since the initial insult is alveolar hypoxia, reversal of the vascular remodeling can be observed following increase of oxygen back to normal level (21% O_2). However, the reversal process can be slow and incomplete following long-term hypoxia exposure (Rabinovitch et al., 1981, Li et al., 2004).

Chronic hypoxia with sugen (SU5416)

Chronic hypoxia combined with sugen, a VEGF inhibitor, was developed to recapitulate more severe and obliterative vascular lesions in human PH (Taraseviciene-Stewart et al., 2001). In rats, a single dose of subcutaneous SU5416 (20 mg/kg) followed by 3 weeks of hypoxia induces proliferation of apoptosis-resistant ECs accompanied by near-complete lumen obliteration of medium-sized and intra-acinar arteries. In addition, when the rats are re-exposed to normoxia for an additional 10-11 weeks, plexiform-like lesions and decreased cardiac output can be observed (Oka et al., 2007, Abe et al., 2010).

In contrast, a single dose of SU5416 combined with chronic hypoxia is not enough to induce severe PH lesions in mice, possibly due to different metabolism in mice compared to rats. It has been reported that 20 mg/kg of SU5416 once weekly, subcutaneously or intraperitoneally, combined with 3 weeks of chronic hypoxia can be used to induce severe PH with concentric neointimal thickening and occlusion in arterioles in mice (Ciuclan et al., 2011, Wang et al., 2013). However, following return to normoxia there is no PH progression but instead

decreased medial wall thickening and decreased right ventricular hypertrophy. (Wang et al., 2013, Vitali et al., 2014).

Monocrotaline

Monocrotaline is a toxic pyrrolizidine alkaloid derived from the plant *Crotalaria spectabilis*. Following processing by cytochrome P-450 (CYP3A4) in the liver it becomes a reactive metabolite (Wilson et al., 1992, Kasahara et al., 1997), which enters the circulation and causes endothelial dysfunction (Schultze and Roth, 1998) and acute pulmonary vasculitis (Chesney and Allen, 1973). In rats a single dose of subcutaneous or intraperitoneal monocrotaline (40-80 mg/kg) induces vascular wall thickening, PH and right ventricular hypertrophy, but no occlusive vascular lesions. Notably, myocarditis (Akhavein et al., 1985, Gomez-Arroyo et al., 2012b) with prominent lymphocyte infiltration has also been found in this model. High pulmonary artery pressure may therefore not be the only cause of morbidity and mortality in this model.

It should be noted that monocrotaline is a toxin which causes toxicity not only to pulmonary vessels and myocardium, but also to liver (Perazzo et al., 1999) and neurons (Silva-Neto et al., 2010). In addition, some pharmacological agents which can reverse monocrotaline-induced PH in rats have no effect in human PH (reviewed in Stenmark et al., 2009). This model is therefore suboptimal for testing new drugs.

Other preclinical models

In addition to the chronic hypoxia and monocrotaline model, genetically modified mouse models of PH are available, in which mice develop PH spontaneously due to either loss of function or overexpression of a specific gene. These models enable us to study distinct molecular pathways *in vivo* and have been discussed in the following reviews (Stenmark et al., 2009, Ryan et al., 2011, Gomez-Arroyo et al., 2012a).

Unfortunately, the perfect animal model for human PH is not yet available. However, some pathobiological responses in the animals share common pathways with human PH thus providing us clues in the search for effective treatments. Table 2 summarizes the similarity of each commonly used animal model of PH.

Table 2. Comparisons of pathophysiologic findings in the commonly used PH models

Stimuli	**Chronic hypoxia**	**Chronic hypoxia**	**Chronic hypoxia with sugen**	**Chronic hypoxia with sugen**	**Monocrotaline**
Species	Mouse	Rat	Mouse	Rat	Rat
Vascular remodeling					
Neointima	No	No	Yes	Yes	Yes
Medial hypertrophy	Yes	Yes	Yes	Yes	Yes
Adventitial fibrosis	Yes	Yes	Yes	Yes	Yes
Plexiform formation /obliterative lesions	No	No	Yes	Yes	No
Perivascular inflammation	Yes	Yes	Yes	Yes	Yes
Increased RVSP	Yes	Yes	Yes	Yes	Yes
Disease progression					
RV hypertrophy	Yes	Yes	Yes	Yes	Yes
*RV dysfunction /RV failure**	?	Yes	Yes	Yes	No (40 mg/kg)
Disease reversibility following removal of stimuli	?	Irreversible RV dysfunction (Bonnet et al., 2004)	Reversible (Vitali et al., 2014)	Irreversible (Abe et al., 2010)	40 mg/kg: reversible (Ruiter et al., 2013) ≥ 60 mg/kg: high mortality

RV: right ventricle; RVSP: right ventricular systolic pressure

?: Not known

*Defined by decreased RV compliance, decreased RV contractility, or decreased cardiac output

RAT MODEL OF NITROFEN-INDUCED CONGENITAL DIAPHRAGMATIC HERNIA (PAPER I)

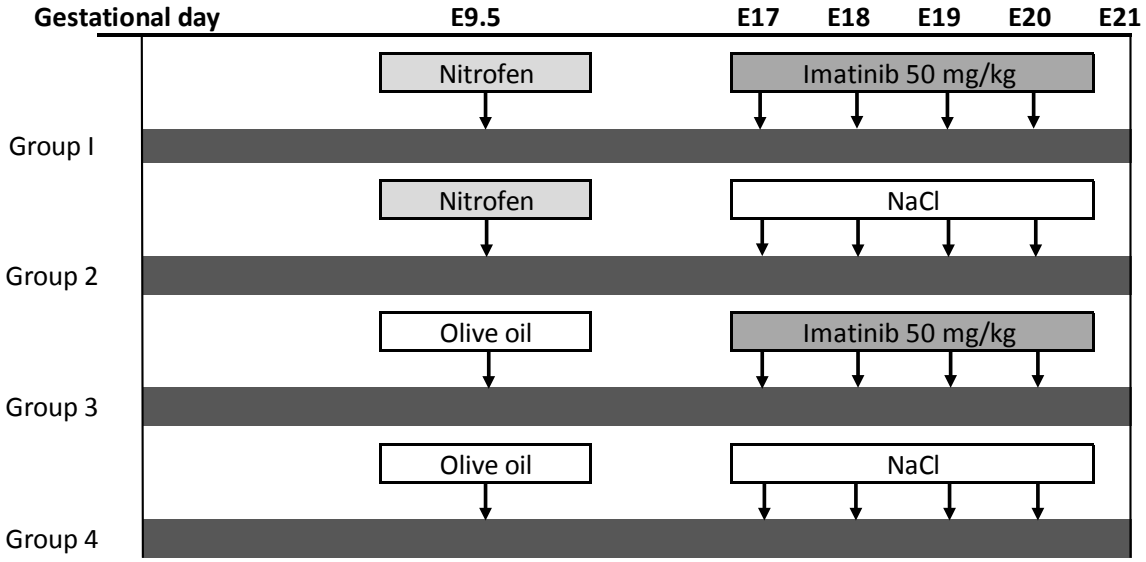
Congenital diaphragmatic hernia (CDH) is a severe life-threatening malformation of the diaphragm characterized by herniation of the abdominal viscera to the chest cavity and accompanied with pulmonary hypoplasia and pulmonary hypertension (Badillo and Gingalewski, 2014). The persistent pulmonary hypertension and pulmonary hypoplasia lead to respiratory failure in the newborns with CDH.

The nitrofen-induced rat model of CDH is the most commonly used preclinical model for studies of the pathogenic mechanisms. The offspring of nitrofen treated rats have diaphragmatic defects, pulmonary hypoplasia, and pulmonary vascular remodeling similar to human CDH (Costlow and Manson, 1981, Newell et al., 1998, Okoye et al., 1998, Luong et al., 2011). Although the actual mechanism of CDH is not fully understood, it has been shown that nitrofen (2,4-dichloro-1-(4-nitrophenoxy)benzene), a teratogenic herbicide, disturbs the retinoic acid pathway during organogenesis (Greer et al., 2003), and inhibits growth of the pleuroperitoneal folds (Clugston et al., 2010).

Clinically, newborns with CDH have a high risk of acute right heart failure when the PH is not responsive to inhaled nitric oxide under optimized ventilatory support (Mohseni-Bod and Bohn, 2007). In addition, continued PH or recurrence of PH after surgical correction causes significant morbidity and mortality in CDH patients (Peetsold et al., 2009).

A previous case report from our collaborators had described successful use of imatinib in a neonate with CDH and severe PH (Frenckner et al., 2008). We therefore hypothesized that antenatal treatment targeting pulmonary vascular remodeling/PH in CDH could be beneficial and possibly reduce the need for extracorporeal membrane oxygenation (ECMO) in the neonatal period. The aim of paper I was to explore the effect of prenatal PDGF inhibition on pulmonary vascular remodeling in CDH. CDH was induced by oral administration of nitrofen (100 mg in 1 ml of olive oil) to pregnant rat dams on gestational day 9.5 (Kluth et al., 1990). At birth, approximately 60%-70% of the pups developed CDH. Imatinib was used for PDGF inhibition (Figure 16).

(A) **Pregnant Sprague-Dawley rats**



(B)

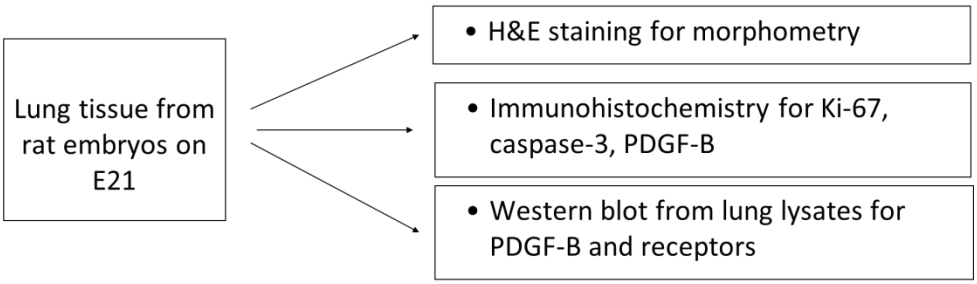


Figure 16. *(A) Timeline for the use of imatinib in paper I. (B) Methods for analysis of pulmonary vascular remodeling.*

CHRONIC HYPOXIA MODEL (PAPER II AND PAPER IV)

Chronic hypoxia exposure (10 % O_2) is a relevant model for the hypoxia-induced vascular remodeling seen predominantly in group III PH (secondary to other lung diseases). However, since the aim of many studies is to explore the mechanisms which govern the increase in vascular muscularization seen in PH, independent of underlying etiologies, chronic hypoxia model is still widely used due to its simplicity and reproducibility. It is often beneficial to use a mouse model because of access to relevant transgenic mouse strains, lacking or overexpressing a gene of interest. A schematic diagram for the setup of the hypoxic cabinet (Coy laboratory products, USA) used in paper II and paper IV is shown below (Figure 17).

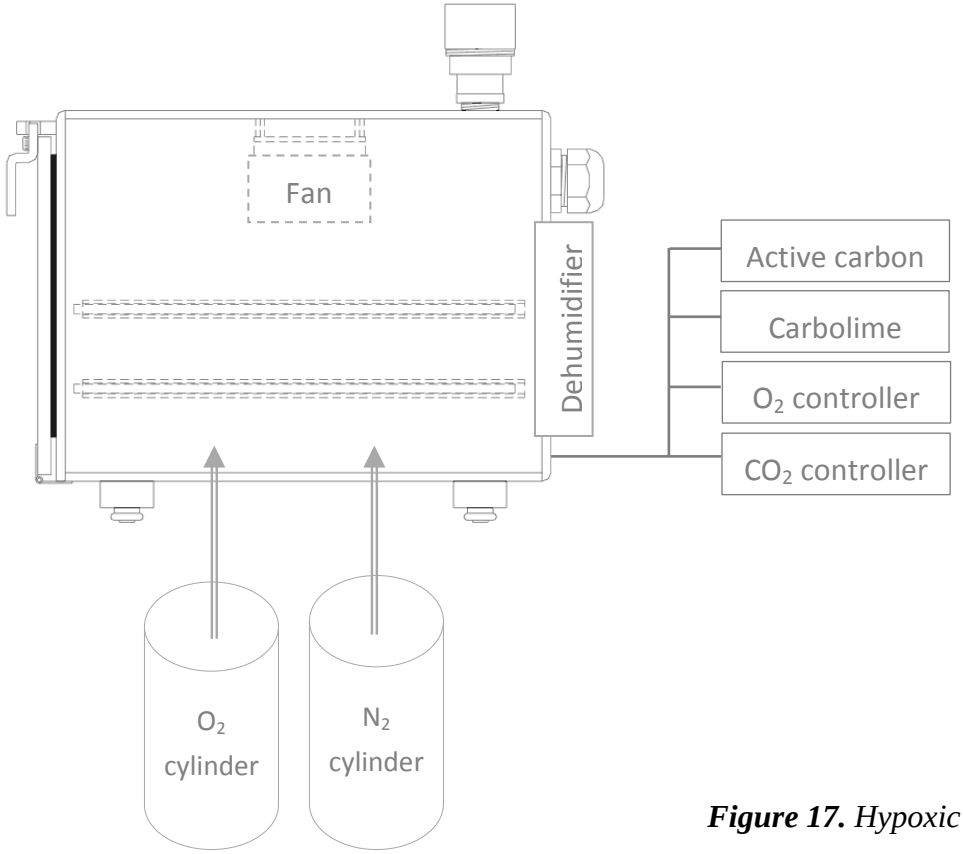


Figure 17. *Hypoxic cabinet*

Even though the hypoxia-induced PH model has been used for more than 30 years, the data regarding the exact time course of pulmonary vascular remodeling and right ventricular remodeling is still lacking. This may be owing to the difference in the response to hypoxia between species and there are also variances between strains (Tada et al., 2008). For example, calves usually develop more severe PH than rats, and rats develop more severe PH than mice (Stenmark et al., 2009). In addition, age and gender are also important factors (Rabinovitch et al., 1981). Younger mice and rats usually develop higher PH than elder ones and males have higher RVSP than females. The approximate timing of major events during development of hypoxia-induced PH is described below (Table 3).

Table 3. Timeline of hypoxic PH development for C57BL/6 mice

Day 0	
Day 3-7	Prominent SMC migration and proliferation (White et al., 2010, Sheikh et al., 2014)
Day 7	Gradual decrease of SMC proliferation to baseline level (Sheikh et al., 2014)
3 week	Appearance of increased RVSP and RV hypertrophy (Zhao et al., 2001)
4-6 week	Slow increase of RVSP and RV hypertrophy (Li et al., 2009)
7-10 week	PH reaches a plateau, no deterioration? (Our pilot experiments)

To confirm that the mice have been adequately exposed to hypoxia, blood samples can be drawn from the right ventricle to check hemoglobin levels. Increased hemoglobin levels indicate the presence of physiologic polycythemia and successful hypoxia exposure. To evaluate the severity of PH, we routinely perform morphometric analysis of pulmonary vascular remodeling/increase in muscularization, RVSP measurements, and obtain the Fulton ratio (defined by the dry weight ratio of the right ventricle to the left ventricle plus interventricular septum, as an index of right ventricular hypertrophy). Several studies of genetically modified mice have revealed that these three parameters, increased vascular muscularization, increased RVSP, and increased Fulton ratio, are not always present concomitantly following chronic hypoxia (Stenmark et al., 2009, Gomez-Arroyo et al., 2012a).

Measurement of right ventricular systolic pressure

Following hypoxia treatment we induce anesthesia with 3% isoflurane and 1.5% isoflurane is maintained for right heart catheterization. A closed-chest approach is used since open-chest methods require endotracheal incubation and mechanical ventilation, factors which would influence the pressure measurements. Following right jugular vein cannulation, a 1.4 French Micro-Tip pressure catheter connected to a transducer (Model SPR-671, Millar) is advanced into the right ventricle. By observing the shape of the pressure curve on the display of the data acquisition system we can determine the localization of the catheter tip and be sure that it has passed the right atrium and is correctly placed in the right ventricular chamber. Continuous pressure recording (for 2-3 min) for each mouse is necessary for reliable data collection for further analysis.

It is important to maintain the same anesthetic conditions and the same procedure duration (usually 10 min) for each mouse. Higher concentration of the anesthetic agent or longer duration will reduce the RVSP and induce bradycardia.

Tissue preparation and morphometric analysis

Mice have four lobes in the right lung and one big lobe in the left. Therefore, we prefer to use the left lung for histology and the right lung for RNA and protein extraction. For histology, the pulmonary circulation is flushed with PBS through the right ventricle until the blood completely comes out from the opening of thoracic aorta. The lungs are then perfusion fixed with 4% zinc-formaldehyde via the right ventricle, while being kept inflated with zinc-formaldehyde infusion via the trachea.

Since the pulmonary vasculature has multiple branching points like a tree a large number of tissue sections are required for a systematic and reliable morphometric analysis, especially for quantification of distal vascular muscularization.

Imaging the pulmonary vasculature

Several imaging techniques can be applied to study the structural changes in PH. Barium injection combined with micro-CT (computed tomography) (Vitali et al., 2014) has recently been described as a way to visualize pulmonary arterioles three-dimensionally, since barium cannot pass through the capillary network. MICROFIL injection is another alternative for post-mortem analysis (Sullivan et al., 2003, Li et al., 2009). Electron microscopy can also provide extensive structural information on the single cell level (Li et al., 2009).

In vivo X-ray angiography is difficult to perform in mice since the image acquisition rate is not as fast as the murine heart rate (450-750 beats per minute). However, with improved resolution and the possibility to choose a specific phase of contrast filling for image analysis (e.g. arterial phase or venous phase) angiography can be a useful tool for obtaining information from live animals.

Other methods can also be used. For example, cardiac echo is a non-invasive method which can be used to monitor hemodynamic changes over time in the same animal (Thibault et al., 2010). Intravital microscopy makes it possible to observe dynamic cell-cell interactions in the pulmonary microcirculation (Tabuchi et al., 2008). Optical projection tomography, ultrasound biomicroscopy and many other developing techniques will open new imaging possibilities for studies of pulmonary vascular remodeling as well as right ventricular remodeling in murine models of PH (Powell and Wilson, 2012).

MOUSE STRAINS

Perlecan mutant mice (*Hspg2*$^{\Delta 3/\Delta 3}$)

Hspg2$^{\Delta 3/\Delta 3}$ mice were generated by target deletion of exon 3 of the perlecan gene, which results in loss of HS attachment sites in domain I of perlecan (Rossi et al., 2003). The mice were kindly provided by Prof Karl Tryggvason and Prof Raija Soininen, Karolinska Institutet and University of Oulu.

Initially, the mice were generated for studies of kidney physiology and disease. Based on the negative charge and hydrophilic properties of HS, researchers hypothesized that perlecan HS in the glomerular basement membrane would function as a permeability barrier for macromolecules (Kanwar and Farquhar, 1979). However, the *Hspg2*$^{\Delta 3/\Delta 3}$ mice did not have proteinuria (unless aggressively challenged). Instead, the perlecan mutant mice were noted to develop cataract due to increased apoptosis in the epithelial cell layer of the lens.

Hspg2$^{\Delta 3/\Delta 3}$ mice are vital and fertile, whereas a perlecan-null mutation is lethal (Arikawa-Hirasawa et al., 2002, Costell et al., 2002). The *Hspg2*$^{\Delta 3/\Delta 3}$ mice are a useful tool to study the function of N-terminal HS. Previously, our group has used the mice to investigate the role of perlecan HS in intimal hyperplasia and atherosclerosis. In the carotid ligation model, *Hspg2*$^{\Delta 3/\Delta 3}$ mice developed more intimal hyperplasia than wild-type mice, possibly due to defective FGF-2 sequestration (Tran et al., 2004). In atherosclerosis, *Hspg2*$^{\Delta 3/\Delta 3}$ mice cross-bred with apoE null mice developed less atherosclerosis than apoE null control mice, possibly due to decreased lipid retention (Tran-Lundmark et al., 2008).

In paper II, we used *Hspg2*$^{\Delta 3/\Delta 3}$ mice to study the role of perlecan HS in vascular remodeling in PH. To rule out the observed effects associated with other genes within the background, backcross breeding to C57BL/6 was performed for at least 10 generations. Genotyping was routinely performed. At the endpoint of every experiment ear biopsies were also taken for genotyping to confirm the correct genotype.

PDGF-B retention motif knockout mice (*Pdgfb*$^{ret/ret}$)

Pdgfb$^{ret/ret}$ mice were kindly provided by Prof Christer Betsholtz at Karolinska Institutet. The mice were generated for studies of the biological function of the PDGF-B retention motif, a C-terminal region of PDGF-B with repeated basic amino acid residues. At physiological pH, the basic amino acids are protonated and the positive charge can interact with negatively charged HS. This electrostatic interaction is important for maintaining PDGF-B gradients in the ECM during angiogenesis (Lindblom et al., 2003, Abramsson et al., 2007, Stenzel et al., 2009) and may also be of importance in pathological states like PH.

Pdgfb$^{ret/ret}$ mice were generated by insertion of TAA stop codon into exon 6 of the PDGF-B gene, resulting in loss of the C-terminal retention motif (Lindblom et al., 2003). *Pdgfb*$^{ret/ret}$ mice are vital but show impaired pericyte investment into the microvessels, reduced number of renal mesangial cells, albuminuria, and retinopathy. PDGF-B levels in *Pdgfb*$^{ret/ret}$ endothelial cells are lower compared to wild-type control. In paper IV, we used *Pdgfb*$^{ret/ret}$ mice to study the role of the PDGF-B retention motif in vascular remodeling in PH.

PROTEOGLYCAN ANALYSIS

Metabolic labeling of proteoglycans *in vitro*

Metabolic labeling with [^{35}S]sulfate is the most commonly used method for studying endogenous synthesis of proteoglycans, in which cells incorporate [^{35}S]sulfate into the newly produced GAGs. In paper III, human PASMCs were grown for 3 to 4 days in M231 growth medium containing 5% fetal bovine serum and other growth promoting factors. The medium was then changed to DMEM medium containing 0.4% fetal bovine serum for the 6 hours of metabolic labeling with [^{35}S]sulfate. Medium with low content of sulfate (e.g. $MgSO_4$) is recommended to increase the yield of labeled proteoglycans.

Following metabolic labeling, medium samples were collected, and cell layer samples were extracted with solubilization buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.15 M NaCl) containing a protease inhibitor cocktail. The proteoglycans were then purified by DEAE (Diethylaminoethyl)-Sephacel anion-exchange chromatography. During the anion-exchange process the pH was changed from pH 8 to pH 4. In those conditions most proteins lose their negative charge, whereas the GAG chains of the proteoglycans keep the charge and continue to bind to the positively charged gel beads. Thereby we were able to separate proteoglycans from other proteins.

The purified intact proteoglycans were then analyzed by gel filtration chromatography. To analyze GAGs, alkaline treatment with 0.5M NaOH overnight at 4 ºC was used to release GAGs from their core proteins. The samples were then applied on NAP-10 column packed with Sephadex G-25 for desalting. In order to analyze the proportion of HS and CS in the GAG preparations, chondroitinase ABC was used to digest CS/DS. Since a high salt content in the samples will interfere with the enzymatic activity of chondroitinase ABC, the desalting step prior to digestion is necessary.

Gel filtration chromatography

Gel filtration chromatography, or molecular sieve chromatography, is a classic method which can separate molecules by size. Here we used Sephadex G50 to separate undigested HS from CS degradation products following chondroitinase treatment, Superose 6 to analyze length of

GAGs, and Sepharose CL-2B for size separation of intact proteoglycans. The choice of polymer is based on the molecular weight of the molecules in the sample since the separation ranges are different. In the chromatography columns, the molecules of larger hydrodynamic size will pass the porous gel matrix faster, thus appearing in the fractions which elute first, and the smaller molecules will travel through the porous gel matrix slower thus eluting later.

By using Sepharose CL-2B chromatography, we can see changes in hydrodynamic size of intact proteoglycans, which is the result of changes in the size of core protein, changes in the length of GAGs or of the number of GAG chains attached to the core protein (or any combination of the three). The results from Superose 6 chromatography can provide distinct information on changes in the length of GAG chains.

Proteoglycan extraction from lung tissue and Western blot

In order to study the content of specific proteoglycans in lung tissue, proteoglycans were extracted from homogenized tissue with 4M guanidine buffer (4M guanidine HCl, 100 mM sodium sulfate, 100 mM Tris-base, 2.5 mM Na_2EDTA, 0.5% Triton X-100, pH7.0). Before proteoglycan purification by DEAE-Sephacel ion-exchange chromatography, the samples were dialyzed extensively against 7M urea buffer (7M urea, 50 mM Tris-base, 2 mM EDTA, 0.5% Triton X-100, pH 7.5) to remove guanidine. Since guanidine is a chaotropic agent with high ionic strength, it would shield the electrostatic interactions of proteoglycans with the DEAE-Sephacel column.

Following measurement of protein concentration by Bradford protein assay, samples containing equal amounts of protein were used for DEAE-Sephacel ion-exchange chromatography. 8M urea buffer (8M urea, 50 mM Tris-base, 2 mM EDTA, 0.5% Triton X-100, pH 7.5) with 0.25M NaCl was used for equilibration and washing of the column, and the samples were then eluted with 8M urea buffer containing 2M NaCl.

Ethanol precipitation (1.3% potassium acetate in 95% ethanol) at -20 °C for 2 h was then performed 2-3 times to remove urea and detergents. For the first ethanol precipitation, CS (5 μg/mL) was added as a carrier. After air-drying the samples, chondroitinase ABC in chondroitinase buffer (50 mM Tris-HCl, 30 mM sodium acetate, 0.1% BSA, pH 8.0) was added and the samples were incubated at 37 ºC for 3 h. Enzymes for deglycosylation can be chondroitinase ABC, heparinase, or both, depending on the subclasses of proteoglycans of interest. Enzymatic deglycosylation facilitates the entry of proteoglycans into the gels for SDS-PAGE, making it possible to estimate the size of the core proteins.

For Western blot, the samples were reduced and applied on a 3.5% stacking gel with a 4-12% resolving gel for SDS-PAGE. Proteins were then transferred to nitrocellulose

membranes using a Bio-Rad Transblot SD Semi-Dry Transfer Cell. Immunoblotting was performed according to standard protocols.

By looking for the shift in molecular weight following different types of enzyme treatment, it is possible to determine the composition of GAGs attached to a certain proteoglycan. Intact proteoglycans will appear as smear on Western blots because of variable sizes of GAGs. If the smear turns to a discrete band following chondroitinase treatment, this indicates that the GAGs are CS/DS, and not HS.

Summary

In general, to study proteoglycan content in tissue is a challenging task. The available assays are still mainly antibody-based methods, like immunohistochemistry, immunoprecipitation, and Western blot. Immunohistochemistry gives information about localization in the tissue, but is not good for quantification. Western blot is often used for quantification, but requires purification/concentration of the samples and is challenging because of the large size of extracellular proteoglycans. All antibody based methods also requires high specificity of the antibodies and often pretreatment of the tissue or the samples with GAG lyases (so that the antibody can reach the core protein). *In situ* hybridization provides data on gene expression, but may not reveal the presence of different core protein isoforms and does not give information about the sulfation patterns of GAGs. To investigate the biological function of a certain proteoglycan, *in vitro* studies of proteoglycans extracted from the cell cultures and genetically modified mice are the most commonly used methods.

PRIMARY CELL ISOLATION FOR SMC CULTURES

The technique of tissue culture was introduced more than 100 years ago (Harrison, 1907, Rous and Jones, 1916). Since then, cell behavior can be observed in a controlled environment. Methods for isolating and characterizing SMCs have been well described in 1979 (Chamley-Campbell et al., 1979). Outgrowth of explants and enzyme dissociation are the two commonly used methods for obtaining SMCs from various blood vessels. Once SMCs are in subculture or have migrated from explants, they are not in contractile state but have phenotypically modulated and readily proliferate when stimulated with mitogens. The same phenotypic switch can be observed *in vivo* by electron microscopy following vascular injury. A large numbers of studies have revealed extensive alterations in gene expression and protein levels during phenotypic modulation and many factors have been shown to regulate this event. For example, smooth muscle α-actin is present in both synthetic (phenotypically changed) and contractile SMCs, whereas myosin heavy chain and smoothelin appear in the contractile state but not in the synthetic state (reviewed in Yoshida and Owens, 2005, Rensen et al., 2007).

For paper II, we used an enzyme dissociation method to isolate mouse pulmonary artery SMC for studies of cell proliferation and adhesion *in vitro*. This method yields a more heterogeneous, and perhaps more representative SMC population from the blood vessels. In contrast, explant methods will select cells that migrate quickly from the tissue. The main pulmonary arterial trunk and the proximal parts of the right and left pulmonary arteries were isolated under a stereomicroscope and the adventitia was gently removed. The blood vessels were then cut into small pieces and incubated with enzyme dissociation buffer (Ham's F-12 Nutrient Mixture Glutamax medium with 0.5 mg/mL elastase, 2 mg/mL collagenase II, 0.375 mg/mL soybean trypsin inhibitor, and 2 mg/mL BSA) for 2 h at 37 ºC. The cell suspension was then centrifuged, washed in F-12/0.1% BSA, and resuspended in the growth medium (Ham's F-12 Nutrient Mixture Glutamax medium with 20% fetal bovine serum, 50 µg/mL L-ascorbic acid, 50 µg/mL streptomycin, and 50 IU/mL penicillin).

Usually, 6 mice were needed for one cell isolation and pulmonary arteries were pooled. For the initiation of a culture 12-well plates were coated with bovine fibronectin (2.5 µg/cm^2) and the cells were allowed to settle and adhere for 48 h before the first medium change. Growth medium was then changed every second day. In general, it takes 2 weeks to start a subculture. After passage 3, the cells can grow nicely in tissue culture flasks without fibronectin coating. Immunocytochemistry showed that >99% of the cells are positive for smooth muscle α-actin.

CYCLIC CELL STRETCH

In order to study the effect of mechanical strain on ECM protein production, we used a cyclic stretch apparatus kindly provided by Prof Staffan Johansson at Uppsala University.

In preparation for the cell stretch experiments, silicon chambers (4x4 cm^2 inner dimensions) were sterilized and the membranes were coated with bovine fibronectin (10 µg/cm^2) during gentle shaking (Figure 18). Human pulmonary artery SMCs were then plated on the coated chambers and grown to subconfluence. After changing the growth medium to DMEM containing 0.4% fetal bovine serum, 10% of biaxial cyclic stretch was applied to the silicone chambers with a frequency of 0.5 cycles per second.

Following 6 h of cyclic stretch, a portion of cells were stained with trypan blue to assess cell viability. The medium was collected, and the cell layer was lysed for qPCR and Western blot. For metabolic labeling experiments, 50 µCi/mL of [^{35}S]sulfate was added during 6 h of cyclic stretch and the samples were collected for proteoglycan and GAGs analysis.

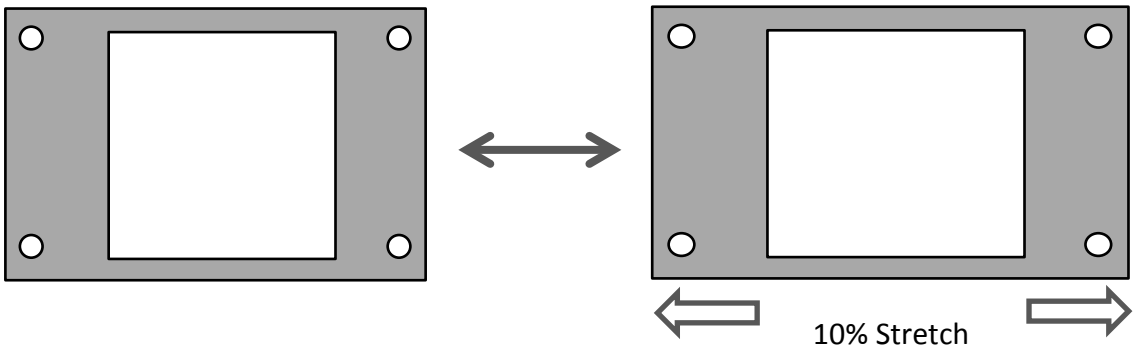


Figure 18. *Chambers for cyclic cell stretch*

For each stretch experiment new silicone membranes were attached to the bottom of the chambers. This procedure helps to acquire reproducible results. We tried to increase the frequency or the duration of the cyclic stretch. However, the cells detach very easily, which makes it difficult to mimic the *in vivo* conditions.

ADDITIONAL METHODS

Other methods used for this thesis like qPCR, Western blot, immunohistochemistry, cell proliferation assay, cell adhesion assay, proximity ligation assay etc. follow standard protocols and were described in papers I-IV.

RESULTS AND DISCUSSION

The overall aim of this thesis was to explore factors that can regulate SMC growth during PH development. In paper I and paper IV, we focused on PDGF-B and its role in pulmonary vascular remodeling. In paper II and paper III, we focused on two proteoglycans, perlecan and versican. We hypothesized that perlecan and versican in the ECM take part in the vascular remodeling process of PH, either directly or indirectly by regulating the bioavailability of growth factors.

PAPER I. ANTENATAL IMATINIB TREATMENT REDUCES PULMONARY VASCULAR REMODELING IN A RAT MODEL OF CDH

CDH is a birth defect associated with pulmonary hypoplasia and persistent pulmonary hypertension. Newborns with CDH display severe respiratory distress at birth. Despite oxygen therapy and optimal mechanical ventilation, some severe cases do not respond to inhaled nitric oxide and need ECMO. The intractable PH makes the medical care for these patients highly challenging.

The abnormal pathological findings in CDH include poor development of the pulmonary vasculature and reduced alveologenesis. Increased vascular wall thickening and vascular hypercontractility are the hallmarks of PH in newborns with CDH. The vascular remodeling persists after surgical correction of diaphragm defect.

Based on the report that the tyrosine kinase receptor inhibitor/PDGF receptor antagonist imatinib can reverse PH in animal models (Schermuly et al., 2005), we hypothesized that imatinib would reduce pulmonary remodeling in CDH by PDGF inhibition. Since PH is a problem already at birth in newborns with CDH, we aimed to evaluate the effect of antenatal imatinib treatment.

In the rat model of nitrofen-induced PH, we showed that oral administration of imatinib to the rat dams resulted in reduced vascular remodeling in the pups. In the imatinib-treated CDH group, the morphological changes in the fetal rat lungs comprised reduced medial wall thickness, decreased proportion of fully muscularized pulmonary arteries, and increased vascular lumen area compared to the CDH group.

We next explored the possible mechanism by evaluating cell proliferation and apoptosis. In CDH, the percentage of Ki-67-positive vascular cells is increased compared to control animals. The imatinib treatment significantly reduced the amount of Ki-67-positive cells, indicating inhibition of cell proliferation. We subsequently examined the protein levels of PDGFs and receptors by Western blot using whole lung homogenates, and by quantification of immunohistochemistry. Both PDGF-B and PDGFR-β were significantly upregulated in

the CDH group compared to controls. The imatinib-treated CDH group revealed a trend towards down-regulation of PDGF-B and PDGFR-β compared to the CDH group, and a significant down-regulation of PDGFR-α was observed.

In summary, we demonstrated that PDGF-B and PDGFR-β were increased in nitrofen-induced CDH. Imatinib acts as an effective anti-remodeling agent by inhibition of cell proliferation. However, we also observed that the control group with imatinib treatment in the absence of nitrofen developed medial wall thinning and increased proportion of distal muscularized vessels. Even though there was no fetal lethality in our experiments, the drug safety still needs to be considered for clinical treatment. Indeed, imatinib has not been approved for PH treatment following clinical trials because of side effects, and it would not be advisable to give it during pregnancy. Studies of more specific pharmacological agents targeting PDGF-B/PDGFR-β are needed.

PAPER II. PERLECAN HS DEFICIENCY IMPAIRS PULMONARY VASCULAR DEVELOPMENT AND ATTENUATES PH

Heparin, the highly sulfated HS, was found to bind FGFs more than 30 years ago (Shing et al., 1984). It has also been shown that HS can either promote or inhibit cell growth (Imamura and Mitsui, 1987). Since then, a large number of studies have revealed dual regulation of heparin-binding growth factors by HSPGs, and provided evidence that HSPGs play a crucial role in fine tuning physiological events (reviewed in Lin, 2004, Bishop et al., 2007, Sarrazin et al., 2011). However, the role of HSPGs in pulmonary vascular remodeling had not been explored.

As mentioned in the introduction, up-regulation of FGF-2 and PDGF-B has been shown in patients with PH and in animal models. Either FGF-2 or PDGF-B inhibition has been demonstrated to reverse experimental PH (Izikki et al., 2009, Schermuly et al., 2005). Since FGF-2 and PDGF-B can interact with HS by electrostatic mechanisms, we aimed to investigate the role of perlecan HS deficiency in PH development using the murine model of hypoxia-induced PH and examined the regulation of these growth factors by perlecan HS.

The initiation of the study was based on previous work from our group where we showed increased intimal hyperplasia in *Hspg2*$^{\Delta 3/\Delta 3}$ mice in a carotid ligation model and increased cell proliferation in *Hspg2*$^{\Delta 3/\Delta 3}$ SMCs *in vitro* (Tran et al., 2004). In that study FGF-2 binding to ECM produced from *Hspg2*$^{\Delta 3/\Delta 3}$ SMCs was significantly reduced compared to wild-type control. The increased intimal hyperplasia in *Hspg2*$^{\Delta 3/\Delta 3}$ mice is likely due to defective sequestration of FGF-2 in the ECM, as a consequence of perlecan HS deficiency.

Therefore, our initial prediction was that *Hspg2*$^{\Delta 3/\Delta 3}$ mice would develop more severe hypoxia-induced PH. However, the results from our pilot experiments did not support this prediction. In addition, *Hspg2*$^{\Delta 3/\Delta 3}$ mice constitutively revealed significantly less muscularization of pulmonary vessels with a diameter of 20-40 μm at baseline under

normoxic condition. To rule out that this phenotype was due to a defect in SMC differentiation, Western blot for several SMC markers was performed and no difference was seen in protein levels of SM-22, myosin heavy chain-11, and smooth muscle α-actin using aortic lysates.

We then hypothesized that the reduction in muscularization was due to loss of mural cells. Staining for several pericyte markers like PDGFR-β, desmin, NG2, SMC α-actin was considerably more sparse in the pulmonary vessels of *Hspg2*$^{\Delta 3/\Delta 3}$ mice compared to wild-type controls (vessel diameters < 50 μm), as examined by confocal microscopy. This suggests loss of pericyte coverage in these vessels.

Notably, the abovementioned vascular muscularization defect only appeared in the distal pulmonary arteries. Histologic examination of the pulmonary trunk and right and left pulmonary arteries was comparable with wild-type. The distal vascular muscularization defect possibly appeared in the embryo during the distal branching process in the pulmonary vascular development (Jones and Capen, 2011). We subsequently checked whether there was any defect in pulmonary vascular branching. Considering the availability of imaging modalities, we then chose X-ray angiography to rule out any arborization defect. *In vivo* X-ray angiography revealed a peripheral filling defect in *Hspg2*$^{\Delta 3/\Delta 3}$ lungs. However, the RVSP in normoxic *Hspg2*$^{\Delta 3/\Delta 3}$ mice was normal.

Based on the abnormal baseline in *Hspg2*$^{\Delta 3/\Delta 3}$ mice, we next planned to investigate hypoxia-induced pulmonary vascular remodeling at different time points (e.g. 3.5 days and 4 weeks).

After 3.5 days of hypoxia exposure, both wild-type and *Hspg2*$^{\Delta 3/\Delta 3}$ mice showed increased perlecan production. Importantly, cell proliferation was significantly reduced in *Hspg2*$^{\Delta 3/\Delta 3}$ pulmonary vessels. Following 4 weeks of hypoxia, *Hspg2*$^{\Delta 3/\Delta 3}$ mice developed PH, but significantly less than wild-type mice. Reduced vascular remodeling and less right ventricular hypertrophy were observed in *Hspg2*$^{\Delta 3/\Delta 3}$ mice.

In order to dissect the molecular mechanisms, we checked the transcripts and protein levels of PDGF-B and FGF-2 and cognate receptors. There were no differences between the genotypes in PDGF-B and PDGFR-β levels, but the hypoxia-induced increase in FGFR1 was inhibited in *Hspg2*$^{\Delta 3/\Delta 3}$ mice following 3.5 days of hypoxia. In addition, we found FGF-2 mRNA and protein levels to be significantly decreased in *Hspg2*$^{\Delta 3/\Delta 3}$ mice compared to wild-type following 4 weeks of hypoxia.

Several studies have reported that perlecan either inhibits or promotes FGF-2 signaling (Aviezer et al., 1994, Pellegrini, 2001, Knox et al., 2002, Katta et al., 2013, Lord et al., 2014). To explore our results further we utilized the FGF ligand and carbohydrate engagement assay (Friedl et al., 2001) to examine the HS-FGF-2-FGFR1 complex formation both on lung sections and *in vitro*. The concept of this assay is that if HS-FGF-2 can form ternary complexes together with FGFR1, the HS in this condition is most likely to promote FGF-2 signaling. On the other hand, when HS-FGF-2 binding cannot form a ternary complex with

FGFR1, HS possibly serves as a sequestration factor for FGF-2 storage. In wild-type mice, we detected fluorescence signals for HS-FGF-2-FGFR1 complexes. However, the signals for perlecan HS deficient mice were significantly reduced. This data suggested that perlecan HS takes part in HS-FGF-2-FGFR1 complex formation. This finding supports our Western blot analysis which showed that hypoxia-induced FGFR1 activation and protein levels were reduced in *Hspg2*$^{\Delta 3/\Delta 3}$ lungs. We speculate that perlecan HS may function as a co-receptor or inhibit proteolysis by ternary complex formation (Figure 19).

(A) (B)

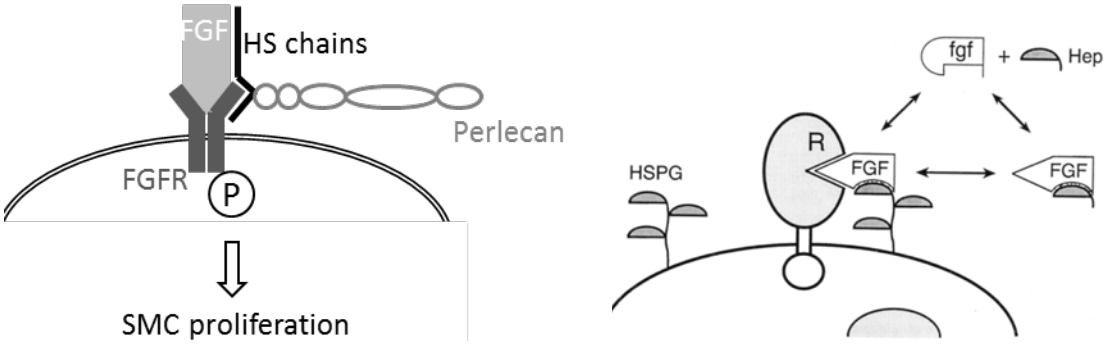


Figure 19. *(A)Possible mechanism of HS-FGF2-FGFR1 interaction. (B)A similar mechanism has been proposed in HS-deficient CHO cells (a inducer-fit model in Yayon et al., 1991). Reprinted with permission.*

In addition to reduced cell proliferation in hypoxic *Hspg2*$^{\Delta 3/\Delta 3}$ lungs, proliferation of pulmonary artery SMCs from *Hspg2*$^{\Delta 3/\Delta 3}$ mice was significantly reduced compared to wild-type control cells under 1 % O_2 with serum stimulation. However, no differences in growth were observed with PDGF-B or FGF-2 stimulation. It should be considered that the recombinant growth factors we used in the cell proliferation assay are not full-length polypeptides containing all HS-binding sites. Despite that they induced biological activities; the recombinant polypeptides may not exert the full function. The electrostatic interactions between growth factors and perlecan HS are possibly not present completely.

To ensure HS deficiency in isolated SMCs from *Hspg2*$^{\Delta 3/\Delta 3}$ mice, we also checked the level of perlecan core protein and HS in the SMC cultures. The results were consistent with our previous finding, which showed reduced HS levels in SMCs from *Hspg2*$^{\Delta 3/\Delta 3}$ mice compared to those from wild-type mice (Figure 20).

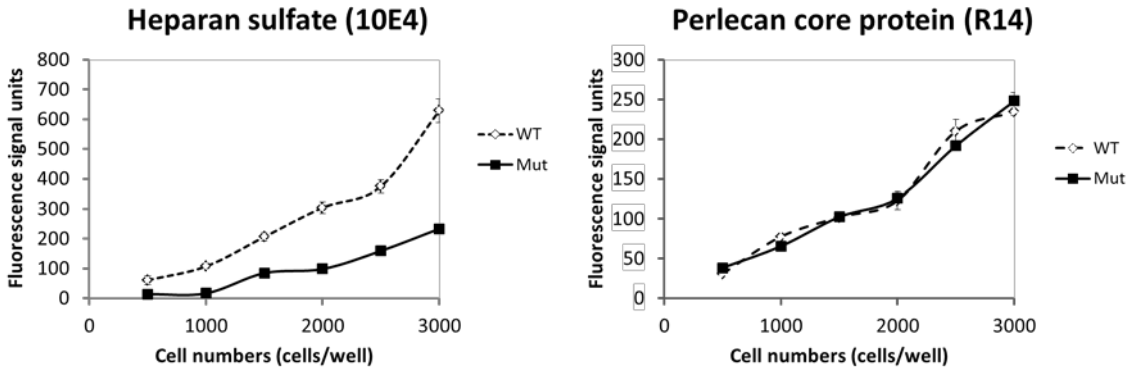


Figure 20. *Results from cell-based ELISA showed that Hspg2$^{\triangle 3/\triangle 3}$ SMCs produced significantly less HS compared to wild-type control cells. No difference in perlecan core protein content was seen. SMCs from wild-type and perlecan mutant mice were plated in 96 well plates with increasing seeding density (500-5000 cells/cm^2) in quadruplicates for 3 days. The cells and pericellular matrix were then fixed with 4% zinc-formaldehyde and blocked with 5% BSA, followed by double-staining with a mouse monoclonal anti-heparan sulfate antibody (clone 10E4) and a rabbit polyclonal anti-perlecan antibody (R14). After incubation with secondary antibodies conjugated with IRDye 680 and IRDye 800 CW, and washing steps, the plates were read using an Odyssey CLx infrared imaging system (LI-COR). The data are presented as mean ±SEM.*

In conclusion, this study highlights the importance of perlecan HS in pulmonary vascular development as well as in the vascular remodeling of PH. Perlecan HS deficiency results in impaired mural cell recruitment to pulmonary vessels and reduced hypoxia-induced PH, partially explained by impaired FGF-2-FGFR1 interaction and inhibition of SMC growth. Future studies on the glycan structure of perlecan HS may help to elucidate the diversity of perlecan HS function.

PAPER III. VERSICAN ACCUMULATES IN VASCULAR LESIONS IN PAH.

Versican is the major CSP in the vascular wall (Theocharis et al., 2001). In systemic blood vessels, versican is upregulated in restenosis and atherosclerosis, where increased versican is associated with SMC proliferation, inflammation and lipid retention (Wight, 2002, Wight and Merrilees, 2004). By metabolic labeling of aortic SMCs, PDGF has been shown to upregulate versican core protein synthesis, change GAG composition, and stimulate elongation of GAGs through protein kinase C and extracellular signal-related kinase (ERK) pathways (Schonherr et al., 1991, Cardoso et al., 2010). However, the role of versican in PH is unknown.

In paper III, we hypothesized that versican would be upregulated in vascular lesions in PH. We used lung tissue samples from patients with PAH of different etiologies, and studied versican levels in various types of vascular lesions. By immunohistochemistry and Western blot, we confirmed that versican is increased in lungs of PAH patients compared to those of healthy donors. Versican prominently accumulates in the vascular ECM in PAH, including in neointima, in medial hypertrophy and plexiform lesions. Accumulation of versican was seen in lungs from patients with idiopathic PAH, PAH associated with scleroderma, and PAH as a result of congenital heart disease.

Notably, versican deposition does not correlate with leukocyte infiltration in the tissue sections we used, as examined by double staining for versican and CD45. However, we cannot exclude a possible role for versican in inflammation in PAH. Vascular remodeling is a dynamic process and the methodology we used here does not allow analysis of different time points during disease progression. The only time point available was the time of transplant, a time when the PH of course is in a very advanced stage.

We then set up *in vitro* studies to explore the factors that can regulate versican production by PASMCs. We first checked the regulation of versican production by different growth factors like PDGF-B and FGF-2, but no differences were found using Western blot.

Based on the finding that versican accumulation was not dependent on the underlying etiology of PAH, we hypothesized that increased mechanical strain and hypoxia could be important factors to promote versican production, considering the pathophysiological environment in pulmonary circulation in PAH. We used metabolic labeling with [^{35}S]sulfate to study proteoglycan synthesis during exposure to mechanical strain/cyclic stretch.

Analyses of [^{35}S]sulfate-labeled proteoglycans synthesized by PASMCs showed that the majority of GAGs in medium samples are CS/DS, whereas GAGs in cell layer samples are HS and CS/DS. During 6 h of 10% biaxial cyclic stretch with a frequency of 0.5 Hz, the hydrodynamic size of proteoglycans was not changed compared to the non-stretch control samples. However, we observed that the peak representing small proteoglycans, possibly biglycan or decorin, disappeared following 6 hr of stretch.

Similar to the metabolic labeling, qPCR and Western blot analysis of PASMCs from stretch experiments did not reveal any increase in versican levels during 6 h of cyclic stretch, while the positive control consisting of aortic SMCs from the same healthy donor showed increased versican gene expression and protein accumulation after 6 h of stretch, consistent with a report in 2001 (Lee et al., 2001). However, increased versican mRNA and protein levels were observed in PASMCs when the samples were collected at 12 h. Versican synthesis by PASMCs thus appeared to be less responsive to mechanical stretch compared to aortic cells and the PASMCs also seemed to produce a lower amount of versican at baseline, which in part may explain why immunostaining and Western blot showed very little versican deposition in the healthy donor lungs.

As mentioned in the “Introduction” section, HIF-1α is an essential element in hypoxia-induced pulmonary vascular remodeling in PH (Ball et al., 2014). It has also been reported that HIF-1α is associated with a high level of proangiogenic progenitor cells in the circulation of patients with PAH (Farha et al., 2011). Recently, a study by Asplund et al. reported that the versican gene, VCAN, has an hypoxia response element, DNA sequence 5'-[AG]CGTG-3', in the promoter region, and that versican gene expression in macrophages is regulated by HIF-1α and HIF-2α (Asplund et al., 2010) . Here we observed increased versican gene expression and protein synthesis in PASMCs following 24h of incubation at 1% O_2, as evaluated by qPCR and Western blot. The association between HIF-1α and versican in PAH awaits further investigation.

In our Western blots, using cell lysates or tissue homogenates, several versican fragments were detected. However, we were unable to differentiate different versican isoforms from versican degradation products. Since Western blot for versican requires DEAE-Sephacel purification, dialysis, and deglycosylation it is difficult to avoid partial degradation, despite the use of protease inhibitors.

In addition, for the lung homogenates, various types of proteases exist in the tissue and the degradation process *in vivo* may be increased in the pathological state. The altered proteoglycan turnover makes it difficult for us to interpret the Western results. Furthermore, SDS-PAGE separates the protein by molecular weight and the antibodies for immunoblotting recognize specific peptide sequences which may be present in some intact isoforms as well as in degradation fragments.

Our finding of versican accumulation in PAH may be associated with aberrant elastin turnover. CS, the part of versican, has been shown to cause shedding of elastin binding protein from the surface of SMCs, and to thereby impair elastic fiber assembly (Hinek et al., 1991). On the other hand, overexpression of V3, the isoform without CS, is able to create an elastin-rich ECM in blood vessels (Merrilees et al., 2002, Merrilees et al., 2011). We speculate that versican accumulation in PH, in combination with increased elastin degradation, results in a water-rich vascular ECM. The environment in the ECM thereby shifts from a hydrophobic state to a hydrophilic state, which facilitates cell migration and proliferation.

PAPER IV. LACK OF PDGF-B RETENTION AMELIORATES HYPOXIA-INDUCED PULMONARY HYPERTENSION

In paper IV, we aimed to evaluate the effect of lack of PDGF-B retention motifs on pulmonary vascular remodeling in the murine model of hypoxia-induced PH. *Pdgfb*$^{ret/ret}$ mice expressing PDGF-B lacking the C-terminal retention motifs were used for the study. *Pdgfb*$^{ret/ret}$ mice are difficult to breed and heterozygous breeding generates too few homozygous knockout animals. Homozygote with heterozygote breeding was therefore used for generating knockouts and *Pdgfb*$^{ret/+}$ heterozygous littermates, which were used as controls. This generated four groups, *Pdgfb*$^{ret/ret}$ normoxia, *Pdgfb*$^{ret/+}$ normoxia, *Pdgfb*$^{ret/ret}$ hypoxia, and *Pdgfb*$^{ret/+}$ hypoxia. Normoxic groups were kept in room air and hypoxic groups were exposed to 10% O_2 for 4 weeks.

Pdgfb$^{ret/ret}$ mice have impaired pericyte investment in the microvessels of the brain, albuminuria and retinopathy (Lindblom et al., 2003). In addition, increased collagen content, endothelial dysfunction and vascular stiffness have been observed in the aorta of *Pdgfb*$^{ret/ret}$ mice whereas the number of vascular SMCs and systemic arterial pressure are normal. Adaptive eccentric cardiac hypertrophy is present in *Pdgfb*$^{ret/ret}$ mice, possibly due to a hyperkinetic circulation (Nystrom et al., 2006).

We examined the gross histology of *Pdgfb*$^{ret/ret}$ lungs by H&E staining and Masson's trichrome staining, and no obvious structural abnormalities or signs of fibrosis were found. The RVSP in *Pdgfb*$^{ret/ret}$ mice was also comparable with that in *Pdgfb*$^{ret/+}$ control mice at baseline (in normoxia).

After 4 weeks of hypoxia, both genotypes developed PH, but the RVSP in *Pdgfb*$^{ret/ret}$ mice was significantly lower. In addition, right ventricular hypertrophy was absent in *Pdgfb*$^{ret/ret}$ mice following chronic hypoxia. In summary, the data suggested that lack of PDGF-B retention reduces hypoxia-induced PH.

We then examined the pulmonary vascular remodeling by immunofluorescence and morphometric analysis. Following 4 weeks of hypoxia, an increase in fully muscularized intra-acinar vessels was seen in *Pdgfb*$^{ret/+}$ mice. *Pdgfb*$^{ret/ret}$ mice however developed a greater proportion of partially muscularized vessels, and no significant increase in fully muscularized vessels. Notably, in the *Pdgfb*$^{ret/ret}$ mice smooth muscle-α-actin-positive cells were present in the alveolar interstitial space in a scattered distribution (Figure 6F in paper IV). This disorganized muscularization pattern may suggest that PDGF-B is not confined to the vascular ECM. Instead, PDGF-B may diffuse freely along the alveolar septae (Figure21).

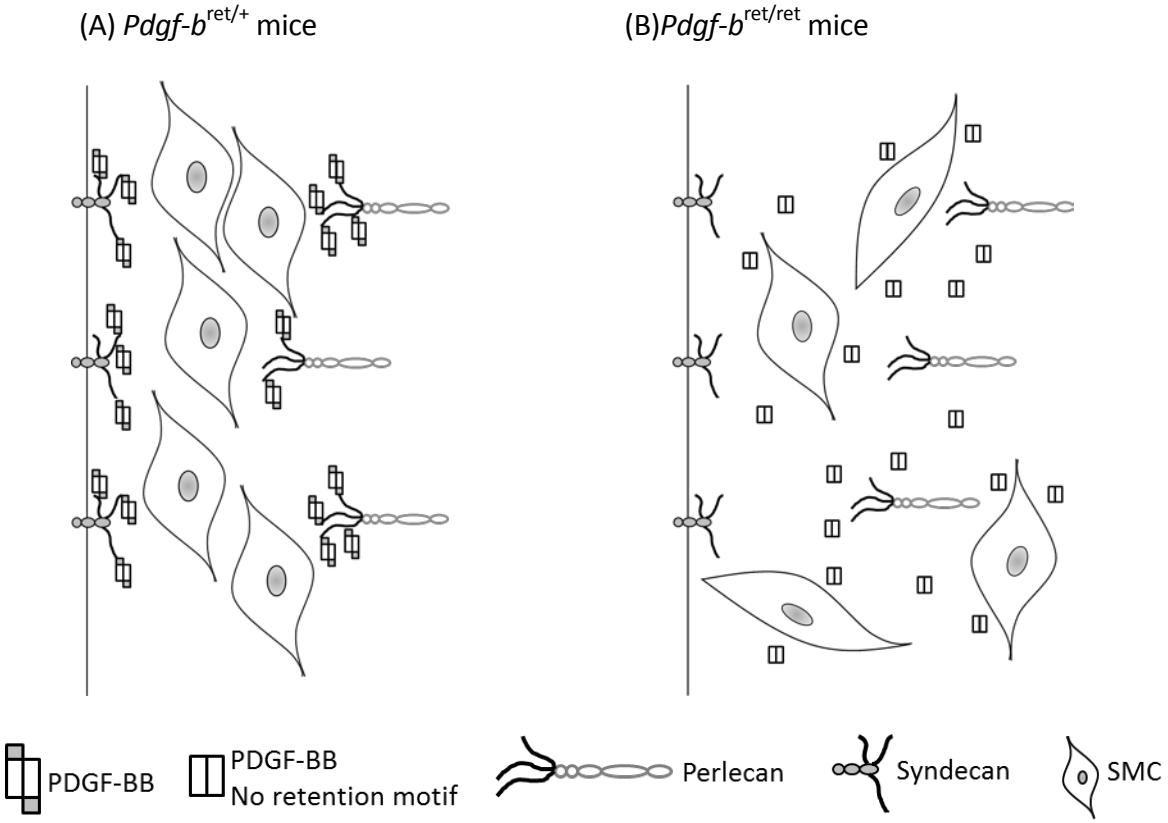


Figure 21. *Working hypothesis for PDGF-B retention in vascular ECM. (A)PDGF-B is confined in the vascular ECM by interacting with HSPGs like perlecan and syndecan. (B)Knockout of PDGF-B retention results in PDGF-B diffusion and SMC disarrangement.*

Since PDGF-B is essential for the recruitment of pericytes and SMCs (Lindahl et al., 1997, Hellstrom et al., 1999), we hypothesize that loss of PDGF-B retention results in defective restraint of mural cell migration. Defective PDGF-B gradients and reduced ability to form fully muscularized vessels following hypoxia may be the reason why *Pdgfb*$^{ret/ret}$ mice seems to be protected from developing PH. To test this hypothesis, more experiments are needed for this project. The first will be to elucidate the identity and morphology of the smooth muscle-α-actin-positive cells in the alveolar septae by high-resolution confocal microscopy and immunofluorescence staining for different pericyte markers and SMC differentiation markers. In addition, it would be very interesting if we can visualize a different tissue distribution of PDGF-B in the *Pdgfb*$^{ret/ret}$ lungs. To count vessels per 100 alveoli in normoxia and hypoxia will also be important since angiogenesis and vascular stability may be affected in the animals. PDGF-B and PDGFR transcripts and protein levels in whole lung lysates also need to be quantified since ECs from *Pdgfb*$^{ret/ret}$ mice have been shown to synthesize reduced levels of PDGF-B *in vitro*. If possible, it would also be of interest to set up an *in vitro* migration co-culture assay for studies of mural cell recruitment.

CONCLUDING REMARKS

In conclusion, the studies in this thesis demonstrate that growth factors and proteoglycans are important regulators of SMC growth during vascular remodeling in PH.

Growth factors, like PDGF-B and FGF-2, promote SMC proliferation and migration and thus drive the vascular repair process in PH. In addition to growth factors, proteoglycans in the ECM participate in PH development. Perlecan is upregulated in the mouse model of hypoxia-induced PH, where perlecan HS regulates FGF-FGF receptor interactions by supporting ternary complex formation. Versican accumulates in the vascular ECM in patients with PAH, which is associated with hypoxia and increased mechanical strain. The overproduction of these proteoglycans may in turn regulate cell phenotype in PH progression, either directly or indirectly by controlling the bioavailability of growth factors and cytokines.

This thesis work reveals several possible targets for anti-remodeling treatment of PH. In addition to PDGF receptor inhibition, the PDGF retention motif, perlecan HS, and versican may provide potential anti-proliferative targets for PH therapy.

FUTURE PERSPECTIVES

As we have demonstrated in paper II, perlecan HS deficiency inhibits PASMC growth and reduces hypoxia-induced PH. We speculate that perlecan HS either serves as a co-receptor or provides a steric hindrance that can protect FGF-2 from proteolytic degradation. However, in the carotid artery, the results are opposite. Previous studies have also reported dual effects of perlecan HS (Knox et al., 2002, Lord et al., 2014), but the exact mechanism which accounts for this dual regulation has not been explored. It is possible that the glycan structure of perlecan HS is cell type-specific and different environmental stimuli may also affect the post-translational modifications. Despite the difficulty to predict the 3-dimensional structure of HS together with the core protein, the modulation of growth factor activity by HS seems to be very specific and effective. It would therefore be of value to analyze the glycan structure of perlecan HS from different cell-types and different tissues, and further dissect the molecular mechanisms in order to design new pharmacological agents.

In paper III we have identified versican accumulation in patients with PAH. Hypoxia and increased mechanical strain may explain the excessive versican deposition in the vascular ECM in PH. More mechanistic studies are needed to understand the regulation by hypoxia and mechanical strain. Further studies on versican function may provide valuable insights into the vascular remodeling process. In addition, the studies of systemic arteries have reported that overexpression of the V3 variant of versican in blood vessels results in a multilayered and elastin-enriched neointima (Merrilees et al., 2011). It will be of interest to test V3 delivery in an animal model of PH and examine the effect on the vascular remodeling process.

In general, studies of pulmonary vascular remodeling in animal model of PH would benefit greatly from better non-invasive imaging modalities for hemodynamic measurements at serial time points. Changes of blood flow in the pulmonary circulation and in the right heart chamber are of importance for the tissue remodeling process. However, this information is largely unknown for the commonly used animal models. High throughput histologic analysis for systematic evaluation of vascular remodeling in the pulmonary vascular tree is also needed for obtaining more precise data, which is especially important for the detection of subtle changes in genetically modified mice.

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“If I have seen further it is by standing on the shoulders of giants.”

Isaac Newton